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(54) Title: ASSAY TECHNIQUES BASED ON GROWTH STAGE DEPENDENT EXPRESSION INC. ELEGANS

(57) Abstract: This invention is directed to new methods to perform assays with nematodes, and more particularly with microscopic nematodes such as *C. elegans*. In particular, the invention provides methods based on the use of growth-stage specific promoters to drive growth-stage specific gene expression.

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Assay techniques based on growth stage dependent expression in *C. elegans*.

This invention is directed to new methods to perform assays with nematodes, and more particularly with microscopic nematodes such as *C. elegans*.

5 The assay techniques described herein may *inter alia* be used for a variety of purposes, such as the discovery and development of compounds for pharmaceutical, veterinary and/or agrochemical use, the selection and isolation of mutant nematode strains, and may also be used for the specific expression of desired amino acid sequences, such as polypeptides and/or proteins, at various growth stages of the
10 nematodes, among others.

Other aspects, embodiments, applications and advantages of the present invention will become clear from the further description hereinbelow.

General techniques and methodology for performing *in vivo* assays using the nematode worm *Caenorhabditis elegans* (*C.elegans*) - i.e. as a model organism for
15 higher multicellular animals - have been described in the art, most notably in the following applications by applicant: PCT/EP99/09710 (published on 15 June 2000 as WO 00/34438); PCT/EP99/04718 (published on January 15, 2000 as WO/00/01846); PCT/IB00/00575 (published on October 26, 2000 as WO 00/63427); PCT/IB00/00557 (published on October 26, 2000 as WO 00/63425); PCT/IB00/00558 (published on
20 October 26, 2000 as WO 00/63426); as well as for instance PCT/US98/10080 (published on 19-11-1998 as WO 98/51351), PCT/US99/13650, PCT/US99/01361 (published on 29-07-1999 as WO99/37770), and PCT/EP00/05102.

As described in these applications, one of the main advantages of assays involving the use of *C.elegans* is that such assays can be carried out in multi-well plate
25 format (with each well usually containing a sample of between 2 and 100 worms) and - also because of this - may also be carried out in an automated fashion, i.e. using suitable robotics (as are described in the aforementioned applications and/or as may be commercially available). This makes assays involving the use of *C.elegans* ideally suited for the screening of libraries of chemical compounds, in particular at medium to high
30 throughput. Such automated screens may for instance be used in the discovery and/or development of new compounds (e.g. small molecules and/or small peptides) for pharmaceutical, veterinary or agrochemical/pesticidal (e.g. insecticidal and/or nematocidal) use.

Some other advantages associated with the use of *C.elegans* as a model organism (e.g. in the assay techniques referred to above) include, but are not limited to:

- *C.elegans* has a short life-cycle of about 3 to 4 days.

This not only means that these nematodes (and suitable mutants, transgenics and/or stable lines thereof) can be cultivated/generated quickly and in high numbers, but also allows assays using *C.elegans* to test, in a relatively short period of time and at high throughput, the nematode worms over one or more, and up to all, stages of life/development, and even over one or more generations. Also, because of this short life span, in *C.elegans* based-assays, compounds may be tested over one or more, and up to essentially all, stages of development, without any problems associated with compound stability and/or (bio)availability;

- *C.elegans* is transparent, allowing -with advantage- for visual or non-visual inspection of internal organs and internal processes, and also the use of markers such as fluorescent reporter proteins, even while the worms are still alive. Also, as further mentioned below, such inspection may be carried out in automated fashion using suitable equipment such as plate readers;

- *C.elegans* is a well-established and well-characterized model organism. For example, the genome of *C.elegans* has been fully sequenced, and also the complete lineage and cell interactions (for example of synapses) are known. In addition, *C.elegans* has full diploid genetics, and is capable of both sexual reproduction (e.g. for crossing) as well as reproduction as a self-fertilizing hermaphrodite. All this may provide many advantages, not only for the use of *C.elegans* in genetic and/or biological studies, but also for the use of *C.elegans* in the discovery, development and/or pharmacology of (candidate) drugs for human or animal use.

- Techniques for transforming, handling, cultivating, maintaining and storing (e.g. as frozen samples, which offers great practical advantages) *C.elegans* are well established in the art, for instance from the handbooks referred to below. For example, *C.elegans* may be used as a one or more samples with essentially fully isogenic genotype(s).

Generally, in the assays described above, the nematodes are incubated in suitable vessel or container - such as a compartment or well of a multi-well plate - on a suitable medium (which may be a solid, semi-solid, viscous or liquid medium, with liquid and viscous media usually being preferred for assays in multi-well plate format). The nematodes are then contacted with the compound(s) to be tested, e.g. by adding the

compound to the medium containing the worms. After a suitable incubation time (i.e. sufficient for the compound to have its effect - if any - on the nematodes), the worms are subjected to a suitable detection technique, i.e. to measure/determine a signal that is representative for the influence of the compound(s) to be tested on the nematode
5 worms, which may then be used as a measure for the activity of the compound(s) in the *in vivo* assay. Often, such a signal will be based on and/or derived from (changes in) at least one biological, phenotypical, behavioural and/or biochemical property of the worm, such as drinking, pharynx pumping, movement, egg laying, mating or defecation (vide for instance PCT/IB00/00575). These properties are also generally referred to as
10 "(biological) read outs" of or for the assay.

Often, in particular for automated assays, such a detection technique involves a non-visual detection method (as further described in the applications mentioned above), such as measurement of fluorescence or another optical method, measurement of a particular marker (either associated with worms or associated with the medium) such as
15 an autonomous fluorescent proteins (AFP) for example green fluorescent protein (GFP), aequorin, alkaline phosphatase, luciferase, Beta-glucuronidase, Beta-lactamase, Beta-galactosidase, acetohydroxyacid, chloramphenicol acetyl transferase, horse radish peroxidase, nopaline synthase, or octapine synthase. For example, for automated assays carried out in multi-well plates, so called (multi-well) "plate readers" may be used
20 for detecting/measuring such a signal.

For a further description of the above and other assay techniques involving the use of nematodes as a model organism, reference is made to the prior art, such as the applications by applicant referred to above.

For general information on *C.elegans* and techniques for handling this nematode
25 worm, reference is made to the standard handbooks, such as W.B. Wood et al., "The nematode *Caenorhabditis elegans*", Cold Spring Harbor Laboratory Press (1988) and D.L. Riddle et al., "C. ELEGANS II", Cold Spring Harbor Laboratory Press (1997), and *Caenorhabditis elegans*, Modern Biological analysis of an organism: ed. by H. Epstein and D. Shakes, Methods in Cell Biology, Vol 48, 1995

30 Although the assay techniques described in the prior art mentioned above demonstrate the usefulness of *C. elegans* in a range variety of *in vivo* assays and for a variety of different purposes, there is an ever continuing need to develop further *C.elegans* based assays, in order to further broaden and expand the applicability of this model organism in drug discovery, development, testing and pharmacology.

The present invention provides such assay techniques, which, in addition to the advantages described hereinbelow, again have all the general advantages associated with the use of *C.elegans* as already described above.

In particular, the invention provides such assays, which are based on (changes 5 in) growth and/or development of the nematode as the biological read out.

The invention is *inter alia* based on the fact that the nematodes used show a number of very distinct stages of development, e.g. from egg to the subsequent development stages referred to as embryonic (early, mid, late), L1, L2, L3 and L4, respectively, to adult. In addition, and mainly depending on environmental factors such 10 as the absence of food, temperature, population and/or certain pheromones, the nematodes may optionally go into a specific and very distinctive stage called the "dauer-state" (which, although an optional stage of development, for the purposes of the present application is also considered a stage of life/development of the nematode).

Thus, more in particular, the present invention provides assay techniques which 15 have been specifically designed to make use of such transition(s) by *C.elegans* from a first stage of development to another (i.e. second, and usually subsequent) stage of development as a biological read out.

The invention is also based on the fact that certain genes within the genome of the nematodes are expressed only during some of these stages of development of the 20 nematodes, but not during some other stages. This is essentially because the promoters associated with these genes drive the expression of these genes in a manner that is dependent on the stage of development.

Some non-limiting examples of such "*development-dependent*" promoters, as 25 well as the specific stage(s) of development in which they drive expression of their associated gene(s), are mentioned in Table 1 below. Others may be found in the handbooks referred to above.

Table 1: Promoters with growth stage dependent expression in *C. elegans*

glp-1	Very early embryonic stage	WBG* 13(2):22 (1feb, 1994)
unc-54	Mid-late embryonic stage	WBG 13(2):22 (1feb, 1994)
myo-2	Mid-late embryonic stage- adult	WBG 13(2):22 (1feb, 1994)
vit-2	Adult	WBG 13(2):22 (1feb, 1994)
lin-28	Embryonic-late L2	WBG 14(5):56 (1feb, 1997)
lin-4	Late L1-adult	<i>C.elegans</i> II :501-518
lin-14	Late embryonic- mid L1	WBG 11(3):46
col-7	L4-early adult	WBG (11)4:61
col-19	L4-early adult	WBG (11)4:61
col-17	Late embryonic-L3	WBG (11)4:61
ctl-1	Dauer	Nature 399:162-166
sod-3	Dauer	FASEB 13: 1385-1393

WBG*: worm breeders gazette

5 One promoter of particular interest for the purposes of the present invention is the vit-2 promoter, which specifically induces expression in the adult stage of the worm, and does so in a very stringent manner. The regulation and gene expression of the vitellogenin gene of *C. elegans* designated vit-2 promoter is well known, and the promoter region has been analyzed in detail. (MacMorris et al., Mol. Cell. Biol., 1992, 10:1652-1662; MacMorris et al., Mol. Cell. Biol., 1994, 14:484-491; Greenspoon et al., worm breeder's gazette, 1988, 10:25).

In the present invention, the "development-dependent" promoters referred to above are used to provide transgenic (strains of) nematode worms, which strains can be used in the assay techniques of the invention.

15 Thus, although promoters that may provide for development-dependant expression in *C.elegans*, as well as transgenic *C.elegans* lines that use such promoters for development-dependant expression in *C.elegans* have been described in the art, so far, such promoters and transgenes have not yet been used in the art in (the design of)

assay techniques, in particular in (the design of) automated, high-throughput assay techniques.

Generally, to accomplish the present invention, the inventors have constructed transgenic nematodes which contain a growth stage dependent promoter operationally linked to a marker gene, and have used this transgenic nematode to develop assays which can be configured for a high throughput setting. The speed of growth or the passage in one of the growth stages which is monitored by the expression of the marker gene which is only expressed in a specific growth stage is then the criteria for selection. Mutant nematodes, and chemically treated nematodes are known to show growth delay, or even growth stage growth arrest. So this method allows for the selection of nematodes which grow faster or slower than the reference nematode. Particular descriptions and examples below are included to clarify this new method.

Thus, in a first aspect, the invention relates to a method for determining the influence of at least one exogenous factor on the development and/or growth of a sample of nematode worms, said method comprising:

a) providing a sample of nematode worms,

which nematode worms contain a marker gene operably linked to a promoter,

which promoter is capable of driving the expression of the marker gene in the nematode worms such that the marker gene is not expressed in at least a first development stage of the nematodes, but is expressed in at least a second development stage of the nematodes (different from the first life stage);

b) exposing said sample of nematode worms to at least one exogenous factor;

c) maintaining/cultivating said sample of nematode worms in a suitable medium, optionally over one or more life stages and/or generations;

d) subjecting the sample of nematode worms to at least one detection technique that is capable of detecting the signal generated by the marker gene (if expressed).

The nematodes used are preferably from the genus *Caenorhabditis*, such as *Caenorhabditis briggsae* or *Caenorhabditis elegans*.

The sample of nematodes may comprise any suitable number of worms, depending on the size of the container/vessel used. Usually, the sample will comprise between 2 and 500, in preferably between 3 and 300, more preferably between 5 and 200, even more preferably between 10 and 100 nematodes. When the assay is carried out in multi-well plate format, each well usually contains between 15 and 75 worms, such

as 20 to 50 worms. Although not preferred, it is not excluded that a sample may consist of a single worm.

Usually, each such individual sample of worms will consist of worms that - at least at the start of the assay - are essentially the same, in that they are of the same strain, in that they contain the same mutation(s), in that they are essentially of an isogenic genotype, in that they show essentially the same phenotype(s), in that they are essentially "synchronised" (i.e. at essentially the same stage of development; it should however be noted that this stage of development may - and usually will - change during the course of the assay), in that they have been grown/cultivated in essentially the same way, and/or in that they have been grown under and/or exposed to essentially the same conditions, factors or compounds, including but not limited to pheromones, gene suppression (such as by RNAi), gene- or pathway-inducing factors or (small) molecules, and/or gene- or pathway-inhibiting factors or (small) molecules, and/or mutagenesis. However, in its broadest sense, the invention is not limited thereto.

In step a), when the sample of nematodes is initially provided, it is preferably such that the nematodes are essentially all in the first development stage.

Preferably said first development stage is such that it precedes the second development stage, in which said first development stage and said second development stage may or may not be separated (i.e. in time) by one or more further, intermediate development stages. For example, the first development stage may be L1, and the second development stage may be adult, with L2, L3, and L4 being intermediate development stages.

Preferably, the first development stage is chosen from eggs, an embryonal stage, L1, L2, L3, L4, or dauer; with eggs, embryonal stages, L1, L2 and dauer being particularly preferred, and L1 being the most preferred.

The second development stage is preferably a development stage subsequent to the first development stage (which may also be, if the first stage is dauer, any stage following escape from dauer) and is preferably chosen from L4, adult or dauer, and more preferably from adult or dauer, dependant on the choice of the first development stage. However, as can also be seen from Table 2 below, which lists some preferred combinations of first development stage, second development stage and promoter, the invention is not limited strictly thereto.

Table 2: some preferred combinations of first development stage, second development stage and promoter.

Promoter	First stage	Second stage
glp-1	L1, L2, L3, L4, dauer, (adults)	very early embryonic stage (eggs)
unc-54	L1, L2, L3, L47 dauers,(adults)	mid-late embryonic stage (eggs)
myo-2	Very early eggs	mid-late embryonic stage-adult
vit-2	Eggs, L1, L2, L3, (L4, dauer)	Adult
lin-28	L4, dauer, adult (L3)	Embryonic-late L2
lin-4	Eggs	late L1-adult
lin-14	L3,L4, adult, dauer, (L2)	late embryonic- mid L1
col-7	Eggs, L1, L2, dauer, (L3)	L4-early adult
col-19	Eggs,L1, L2, dauer, (L3)	L4-early adult
col-17	Adult, dauer, (L4)	Late embryonic-L3
ctl-1	Eggs,L1, (L2, L3, L4, adult)	Dauer
sod-3	Eggs, L1,(L2, L3, L4, adult)	Dauer

5

In the assays of the invention, the nematodes may be kept in or on any suitable medium, including but not limited to solid and semi-solid media - but are preferably kept in a suitable liquid or viscous medium (e.g. with a viscosity at the temperature of the assay that is equal to a greater than the viscosity of M9 medium, as measured by a suitable technique, such as an Ubbelohde, Ostwald and/or Brookfield viscosimeter).

Generally, suitable media for growing/maintaining nematode worms will be clear to the skilled person, and include for example the media generally used in the art, such

as M9 (10 X M9 buffer: 30 g KH₂PO₄, 75.212 g Na₂HPO₄. 2H₂O, 50 g NaCl, 10 ml 1M MgSO₄, add up to 1 L), S-buffer (5.9 g NaCl, 50 ml 1M KH₂PO₄, 1ml 5g/L cholesterol, add up to 1 L), and the further media described in the applications and handbooks mentioned hereinabove.

5 The medium may further contain all factors, compounds and/or nutrients as may be required for the survival, maintenance and/or growth of the worms. For this, reference is again made to the prior art, such as the applications and handbooks referred to above. The medium may also contain a suitable source of food for the worms such as bacteria, for example a suitable strain of *E.coli* in a suitable amount, e.g. between 0.001 and 10 10 % (w/v), preferably between 0.01 and 1%, more preferably between 0.1 and 0.2 %, such as about 0.125 % w/v. In one specific embodiment, further described below, said bacteria may also contain or express a double stranded RNA (construct), intended for specific gene down regulation in the nematode worm, e.g. by means of RNA-interference (vide PCT/EP99/04718)

15 The assay may be carried out at a suitable temperature, which may for example be a temperature of between 10 °C and 30 °C, preferably between 20 °C and 27 °C, such as 21, 22, 23, 24, 25 or 26 °C, depending on the specific strain used. The temperature may be kept essentially constant during the course of the assay, and/or may be varied, e.g. within the ranges indicated above.

20 In the method of the invention, the sample of nematodes can be kept - e.g. maintained, grown or incubated - in any suitable vessel or container, but is preferably kept in a well of a multi-well plate, such as a standard 6, 24, 48, 96, 384, 1536, or 3072 well-plate (in which each well of the multi-well plate may contain a separate sample of worms, which may be the same or different). Such plates and general techniques and 25 apparatus for maintaining/ handling nematode worms in such multi-well plate format are well known in the art, for instance from the applications mentioned hereinabove.

The method/assay of the invention is preferably carried out in an automated fashion, e.g. using the equipment and techniques described in the applications mentioned above.

30 In the invention, a nematode strain is used that contains a marker gene that is operably linked to a promoter, which promoter is capable of driving the expression of the marker gene in the nematode worm(s) such that the marker gene is not expressed in at least a first development stage of the nematodes, but is expressed in at least a second development stage of the nematodes (different from the first development stage).

As already indicated hereinabove, such promoters are also referred to herein as "development-dependent" promoters, and some preferred examples have been given above.

A particularly preferred development-dependent promoter is the vit-2 promoter.

5 An operational fusion of a DNA sequence (gene, cDNA) with the vit-2 promoter allows for the expression of this DNA sequence in the adult stage of *C. elegans*, and not in the other life stages of *C. elegans* such as the L1, L2, L3, and L4 larvae stages and the dauer stages.

In the present disclosure, two or more nucleotide sequences, such as a promoter
10 and a marker gene, are considered "operably linked" when they are in a functional relationship with each other. For instance, the development-dependent promoter is considered "operably linked" to the marker gene if said promoter is able to initiate or otherwise control/regulate the transcription and/or the expression of said marker gene, in particular in a development-dependent manner (and in which said marker gene should
15 be understood as being "under the control of" said promoter). Generally, when two nucleotide sequences are operably linked, they will be in the same orientation and usually also in the same reading frame. They will usually also be essentially contiguous, although this may also not be required.

The marker gene may be any gene which, upon expression in *C. elegans* - i.e.
20 under the control of the development-dependent promoter - provides a signal that can be detected, e.g. visually or preferably by the automated, non-visual detection techniques referred to above.

For example, the marker gene may be chosen from green fluorescent protein,
beta-galactosidase, beta-lactamase, luciferase, acetohydroxyacid synthase, alkaline
25 phosphatase, beta-glucuronidase, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase and/or octapine synthase. Other suitable marker genes will be clear to the skilled person, and are for instance described in the applications referred to above.

In a specific embodiment, the gene may be a toxic gene, e.g. a gene that
30 encodes a gene product that is toxic (e.g. lethal) to the nematode. Thus, another application of the invention consists in the conditional expression of putative toxic genes, and in the conditional expression genes, to be expressed in specific growth stage in nematodes such as *C. elegans*. When toxic genes are expressed in nematode at any growth stage, and surely in the early development of the nematode, this will have

dramatic influences on the further development and vitality of the nematode. It may be opportune to express such genes in a particular growth phase of the worm, such as the L1, L2, L3, L4, adult or dauer stages. Such transgenic nematodes have more chance to survive the expression of the toxic gene and may be used for further analysis, for instance in a HTS assay, screening for compounds, mutants, etc. Some preferred, but non-limiting examples of such toxic genes are ataxin, alpha-synuclein, ubiquitin, the tau gene, the huntington gene, the best macular dystrophy gene product, unc-53; others are mentioned in the applications referred to above.

The nematode strain used in the invention may generally be provided by transforming a suitable nematode strain with a nucleotide sequence that comprises the marker gene under the control of the development-dependent promoter. Preferably, said nucleic acid sequence is in the form of a genetic construct, which may be DNA or RNA (and are preferably double-stranded DNA) and which is preferably in a form suitable for transformation of the nematode strain used. For example, it may be in the form of a construct that, upon transformation, is integrated in the genomic DNA of the nematode, and/or may be in a form suitable for independent replication, maintenance and/or inheritance in the nematode. Preferably, the construct is also such that it is capable of independent replication, maintenance and/or inheritance in the (micro-) organism used for cloning, such as *E. coli*. For instance, said genetic construct may be in the form of a plasmid, vector, viron or transposon.

The genetic construct(s) used in the invention may further contain - i.e. besides the nucleotide sequences encoding the development-dependent promoter and the marker gene - one or more further suitable elements of genetic constructs known per se, including but not limited to selection markers and/or elements that may facilitate or increase (the rate of) transformation or integration. These and other suitable elements for such genetic constructs will be clear to the skilled person, also from the applications referred to above.

The constructs of the invention can be provided in a manner known per se, which will generally involve techniques such as restricting and linking nucleic acids/nucleic acid sequences, as will be clear to the skilled person. Reference is made to the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" (2nd.ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989) and F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). The nucleic acids encoding the development-dependent promoters

and marker genes used in the invention have been described in the art and can be provided in the manner described therein.

The nematodes may be transformed with the constructs in any suitable manner, such as micro-injection or ballistic transformation, for which reference is made to the handbooks referred to above, as well as for instance in PCT/EP99/01903 (published as WO 99/49066).

The nematode strain that is transformed with the nucleotide sequence encoding the marker gene/development-dependent promoter - i.e. to provide a nematode strain useful in the assay of the invention - is not particularly limited, and may for instance be any nematode strain known per se, such as wildtype, N2 or hawaiian (CB4856, Hodgkin et al., Genetics 146:149-164, 1997). Also, specific mutant nematode strains or lines and transgenic strains or lines may be used which are particularly suited/adapted for transformation and/or the specific transformation technique used, or if they are desired in the assay.

In one embodiment, before use in the present assays, the nematodes are subjected to random or specific mutagenesis. Thereupon, the different strains resulting from the mutagenesis may be tested in the assay(s) of the invention, and optionally may be compared to the original strain and/or to a(nother) reference strain. This may be done with and/or without exposure to the exogenous factor(s) and may for instance be used to identify genes and/or mutations that influence the development and/or growth of the nematodes, and/or to identify genes and/or mutations which alter or influence the response of the nematodes (i.e. with respect to development and growth) to the exogenous factors. For example, when a mutation in a gene leads to a marked change in development and/or growth (as determined using the assay(s) of the invention), or leads to a markedly different response to the exogenous compound(s), it may be concluded that said gene is involved in development or growth and/or in the response of the nematode to the exogenous factor(s). In this way, the assays of the invention may for instance be used to determine the function of (known or unknown) genes (for instance as part of a functional genomics program) and/or to determine the mode of action of the exogenous factor(s).

In step b), a sample of nematodes containing the marker gene under the control of the development-dependent promoter is exposed to the exogenous factor(s) to be tested. This may be carried out while the nematodes in the sample are (still) in the first stage of development, and/or in any subsequent stage(s) of development. Preferably,

however, the sample of nematodes is exposed to the at least one exogenous factor in at least one stage of development which precedes the second stage of development (however, it should be noted that the invention does not exclude that the sample of nematodes is still in contact with the exogenous factor(s) while the nematodes transit into and/or are in the second stage of development).

For example, the nematodes may be exposed to the exogenous factor(s) in only a single stage of development (such as only in the first stage or only in a subsequent stage that precedes the second development stage), in two or more stages (which may include the first stage, any subsequent stage(s) and/or the second stage), or essentially continuously throughout the duration of the assay.

Thus, generally, the nematodes may be exposed to the exogenous factor(s) during a time of 1 minute up to the entire life (cycle) of the nematodes, and/or the duration of the assay. Usually, a contact time of between 5 minute and 110 hours, preferably between 10 minutes and 80 hours will be preferred.

The total time for the assay will preferably be such that it is sufficient to allow at least one of the nematodes in the sample to transit from the first development stage into a subsequent development stage, and more preferably sufficient to allow at least one of the nematode worms in the sample to enter from the development stage into the second development stage, optionally via any (further) intermittent stages of development

For example, in step c), the sample of nematode worms may be maintained/cultivated for a time such that at least 1%, preferably at least 5%, of the nematode worms present in the sample enter from the first development stage into at least one other/further development stage.

Also, for example, in step c), the sample of nematode worms may be maintained/cultivated for a time such that at least 1%, preferably at least 5%, of the nematode worms present in the sample enter from the first development stage into the second development stage.

Often, the total time for the assay will be at least such that it would allow at least one of the nematode worms present in a reference sample - i.e. a sample not containing any exogenous factor(s) - to enter from the first development stage into the second development stage, optionally via any (further) intermittent stages of development.

For example, for assays from the following first development stage to the following second development stage, the total time of the assay can be as follows: from eggs to adults: 45 to 110 hours; from L1 to adults: 30 to 80 hours; from eggs to L1: 13 to

30 hours; from L1 to L2: 13 to 25 hours; from L2 to L3: 8 to 20 hours; from L3 to L4: 8 to 15 hours; from L4 to adult: 8 to 25 hours; for assays involving dauer as the first or second stage: between 8 and 72 hours (depending on the strain used, temperature and food quality, nematodes will generally enter the L1 growth stage between 13 and 30
5 hours, the L2 growth stage between 24 and 55 hours, the L3 growth stage between 30 and 70 hours, the L4 growth stage between 38 and 85 hours, and the adult stage between 45 and 110 hours, starting from eggs).

During the duration of the assay, the sample may be subjected to the - preferably non-visual - detection method for determining/measuring the expression of the marker
10 gene essentially continuously during the entire duration of the assay, essentially continuously during one part of the duration of the assay (usually the latter part, when the nematodes are considered likely to enter the second stage of development, e.g. during the last 24, 12, or even 6 hours of the duration of the assay), at regular intervals, or any combination thereof.

15 In the assays of the invention, each individual sample of nematode worms will generally be exposed to a single exogenous factor to be tested, at a single amount or concentration; with different samples (e.g. as present in the different wells of the multi-well plate used) being exposed either to different concentrations of the same factor (e.g. to establish a dose response curve for said factor), to one or more different factors (e.g.
20 in the case of compounds for instance are part of a chemical library and/or of a chemical class or series, such as a series of closely related structural analogues; or in case of a library or series of dsRNA constructs for RNAi), or both (e.g. to the same and/or different factors at different concentrations).

It is also within the scope of the invention to expose the (sample of) nematodes
25 to two or more factors - at essentially the same time or sequentially (e.g. with an intermediate washing step) - for example to determine whether the two factors have an effect which is the same or different from both the factors separately (e.g. to provide a synergistic effect or an inhibitory or competitive effect).

Furthermore, it is within the scope of the invention to use one or more reference
30 samples, e.g. samples without any factor(s) present, and/or with a predetermined amount of a reference factor. The invention also includes the use, in an assay, of two or more samples of nematode worms of different strains (e.g. each containing a marker gene under the control of a (different) development-dependent promoter), e.g. to compare (the effect of the factors(s) to be tested on) said different strains.

In one specific embodiment, which is referred to herein as an "FPTP-type assay", each sample of a series of two or more essentially similar samples of nematode worms (e.g. containing the same development-dependant promoter, preferably the same marker gene - although this is not strictly required - and preferably comprised of worms 5 in the same stage of development) is exposed, in essentially the same manner (e.g. time and conditions, but optionally at different concentrations), to (a) different exogenous factor(s), and optionally to one or more reference factors. Thereupon, the order in which the nematodes present in each of these samples enter the second development stage is determined, i.e. by determining the order in which the samples of the series show 10 expression of the marker gene (i.e. which sample shows the expression of the marker gene first, second, third, etc.). Inter alia, this allows the different factors present in each of the samples to be compared and/or ranked according to their influence on the development/growth of the nematode, and also compared to the reference factor(s). This for instance allows the identification of factors with an influence on the nematodes 15 comparable to, or even improved compared to, the influence of the reference factors. Generally, such FPTP-assays will involve determining the (possible) expression of the marker gene in the series of samples essentially continuously, at least during the last 36, 24, 12, or 6 hours of the assay.

Thus, in a specific embodiment, the invention relates to a method for determining 20 the influence of at least a first exogenous factor on the development and/or growth of a sample of nematode worms, said method comprising:

- a) providing at least a first and a second sample of nematode worms,
in which the nematode worms in each sample contain a marker gene operably linked to a promoter,
25 which promoter is capable of driving the expression of the marker gene in the nematode worms such that the marker gene is not expressed in at least a first development stage of the nematodes, but is expressed in at least a second development stage of the nematodes (different from the first life stage);
b) exposing at least said first sample of nematode worms to said first one exogenous factor;
c) maintaining/cultivating said samples of nematode worms in a suitable medium, 30 optionally over one or more life stages and/or generations;

d) subjecting the samples of nematode worms to at least one detection technique that is capable of detecting the signal generated by the marker gene (if expressed);
e) determining the time required for the first sample of nematode worms to show expression of the marker gene (as determined by the signal detected for the first sample in step b)), and preferably also determining the time required for the second sample of nematode worms to show expression of the marker gene (as determined by the signal detected for the second sample in step b)); and/or comparing the time required for the first sample of nematode worms to show expression of the marker gene with the time required for the second sample of nematode worms to show expression of the marker gene.

In one aspect, the second sample of nematode worms will be a reference sample, e.g. a sample of worms that is not exposed to any exogenous factor, or to a known reference factor. The second sample may also be exposed to a second exogenous factor, e.g. to compare the first and the second factor.

Generally, as already indicated above, the assay according to this aspect of the invention will involve the use/testing of a series of samples, e.g. more than 5, preferably more than 10, such as about 6, 24, 48, 96, 384, 1536, or 3072 (i.e. essentially the number of wells of a multi-well plate), each sample being exposed to a different factor and/or to a different concentration of factor (including any reference samples), and the samples than being ranked as described above.

Usually, to allow for a good comparison between the samples/factors, all samples will be essentially similar (as described above) and cultivated/maintained in an essentially similar manner. These FPTP-assays may further be carried out in essentially the manner described herein.

In all the assays described above, the exogenous factor may be any factor the influence of which on the growth/development of nematode worms is to be tested. The exogenous factors may for instance be chosen from small compounds (as defined below), small peptides (as defined below), factors which induce or suppress specific pathways in the worm, factors which induce or suppress (the expression of) specific genes in the worm (such as dsRNAi for RNA-interference), polypeptide and/or proteins, or extracts from natural products(such as plants, animals, fungi, bacteria), amino acids and derivatives, hormones and derivatives, nucleic acids and derivatives.

For the purposes of the present disclosure, a "small molecule" generally means a molecular entity with a molecular weight of less than 1500, preferably less than 1000.

This may for example be an organic, inorganic or organometallic molecule, which may also be in the form or a suitable salt, such as a water-soluble salt.

The term "small molecule" also covers complexes, chelates and similar molecular entities, as long as their (total) molecular weight is in the range indicated above.

5 In a preferred embodiment, such a "small molecule" has been designed according, and/or meets the criteria of, at least one, preferably at least any two, more preferably at least any three, and up to all of the so-called Lipinski rules for drug likeness prediction (vide Lipinski et al., Advanced Drug Delivery Reviews 23 (1997), pages 3-25). As is known in the art, small molecules which meet these criteria are particularly suited 10 (as starting points) for the (design and/or) development of drugs (e.g.) for human use, e.g. for use in (the design and/or compiling of) chemical libraries for (high throughput screening), (as starting points for) hits-to-leads chemistry, and/or (as starting points for) lead development.

In a preferred embodiment, such a "small molecule" has been designed 15 according, and/or meets the criteria of, at least one, preferably at least any two, more preferably at least any three, and up to all of the so-called Lipinski rules for rational drug design (vide Lipinski et al., Advanced Drug Delivery Reviews 23 (1997), pages 3-25). As is known in the art, small molecules which meet these criteria are particularly suited (as starting points for) the design and/or development of drugs (e.g.) for human use

20 Also, for these purposes, the design of such small molecules (as well as the design of libraries consisting of such small molecules) preferably also takes into account the presence of pharmacophore points, for example according to the methods described by I. Muegge et al., J. Med. Chem. 44, 12 (2001), pages 1-6 and the documents cited herein.

25 The term "small peptide" generally covers (oligo)peptides that contain a total of between 2 and 35, such as for example between 3 and 25, amino acids (e.g. in one or more connected chains, and preferably a single chain). It will be clear that some of these small peptides will also be included in the term small molecule as used herein, depending on their molecular weight.

30 Thus, the methods of the invention may in particular be used to test and/or screen (libraries of) such small molecules and/or peptides, in the manner as further outlined herein.

According to another embodiment, the exogenous factor is a factor that suppresses or enhances the expression of one or more genes in the nematodes used. In

one preferred example, this factor may be a dsRNA, which may be used for gene suppression in accordance with well-known RNA-interference techniques. Such dsRNA may for instance be provided to the nematode worms in the manner described in PCT/EP99/04718 (published as WO 00/01846) or PCT/US98/27233 (published as WO 99/32619), e.g. by injection of dsRNA or by feeding of bacteria containing/expressing the dsRNA to the nematode. In this latter embodiment, for example, the effect(s) of the suppression of one or more gene(s) on the growth or development of the nematode worms and/or on the response of other exogenous factors, may be determined.

The nematodes may be exposed to the exogenous factor in any suitable manner, such as by incorporating the exogenous factor in the medium in which the nematode worms are grown/maintained or by incorporating the nematode worms in the food of the nematodes (e.g. in the case of dsRNA for RNAi purposes).

The nematode worms may take up the exogenous factor in any suitable manner, such as by drinking, feeding, soaking, pharynx pumping, or in any other suitable way, e.g. either through (a part of) the gastrointestinal tract, the cuticle and/or through openings in the cuticle, and either through an active or passive uptake mechanism, or any combination thereof.

When the exogenous factor is a compound, it will usually be used in step b) at a concentration of between 0.1 nanomolar and 100 milimolar, preferably between 1 nanomolar and 50 milimolar, more preferably between 10 nanomolar and 10 milimolar, even more preferably between 100 nanomolar and 5 milimolar, in particular between 1 micromolar and 1 milimolar, even more particular between 10 micromolar and 600 micromolar, most particular between 20 micromolar and 500 micromolar, such as about 30 micromolar for compound selection screens and about 300 micromolar for compound resistance screens.

For dsRNA, suitable amounts will be as described in the PCT/EP99/04718 (published as WO 00/01846) or PCT/US98/27233 (published as WO 99/32619).

The assay techniques of the invention may be used for several different applications, some non-limiting examples of which will now be further described.

A first application is to identify and select chemical entities that may be used in the development of pharmaceutical products, veterinary products, and pesticides. In this respect, it should also be noted that the invention may not just be used to identify exogenous factors (such as compounds) which directly influence development and/or growth, but also compounds which influence other behavioural, biological, phenotypical

and/or biochemical processes which in turn influence growth and/or development, such as metabolic processes, feeding/drinking behaviour and/or (other) processes which are controlled by the central nervous system or other nerve cells.

Thus, the invention may also be used to identify compounds which may influence 5 metabolic processes and neuron-controlled processes, not just in nematodes, but also in higher animals including humans and other mammals, for which the nematode is used as a model organism. Thus, the assays of the invention may be used in the discovery and/or development of pharmaceuticals and/or veterinary products.

Also, exogenous factors such as compounds which, in the assays of the 10 invention, retard growth and/or development may find use in the development of novel insecticides or other pesticides (including but not limited to nematocides).

Another application is to identify and select new mutants, and further on isolating 15 the genes which are mutated. This genes and the proteins they encode for are then considered as putative target genes and/or members of biochemical pathways. In a specific variant of this objective, mutants are selected that show resistance to a chemical compound, and once again the final objective is to isolate the mutated gene.

A third possible application is related to the isolation of genes, and the proteins 20 they encode for by dsRNA inhibition (RNAi). The isolated genes and the proteins they encode for are considered as putative target genes, members of biochemical pathways, resistance.

In the development and performance of HTS assays with nematodes, the synchronicity of the animals is of major importance, i.e. nematodes used in the assay need to be at the same growth stage. Although several methods have been developed 25 to grow a culture of nematodes at the same speed, while they are in the same growth stage, aberrations are usual. The present invention also offers a solution to this problem. As the nematodes described in this invention express marker genes at a certain growth stage, the nematodes in a culture at the same growth stage can easily be detected and isolated prior to the HTS assay. Moreover several machines are presently available that allow to select automatically nematodes which have common features (such as 30 expressing a green fluorescent proteins). An example of such machine, generally designated as a worm dispensers or FANS (Fluorescence Activated Nematode Sorter), is provided by UBI (Union, Biometrika, USA). The methods allows the inventors to select nematodes which are in a specific growth stage, such growth stage may for example be, eggs, L1, L2, L3,L4, Adult or dauer growth stage.

In another aspect, the invention relates to the use of a (sample of at least one) nematode worm, which nematode worm contains a marker gene operably linked to a promoter, which promoter is capable of driving the expression of the marker gene in the nematode worms such that the marker gene is not expressed in at least a first 5 development stage of the nematodes, but is expressed in at least a second development stage of the nematodes (different from the first life stage), in a method or assay for determining the influence of at least one exogenous factor on the development and/or growth on a nematode worm.

10 In a particular aspect, the invention relates to the use of a (sample of at least one) nematode worm in an FPTP assay as described above.

The invention will now be further illustrated by means of the following non-limiting Figures and Examples. The Figures show:

- Figure 1: Nucleotide sequence of pGQ1
- Figure 2: Nucleotide sequence of PCLUC6
- 15 - Figure 3: Nucleotide sequence of pGQ2
- Figure 4: Nucleotide sequence of pGN156
- Figure 5: Nucleotide sequence of pGQ3
- Figure 6: Nucleotide sequence of pGQ4
- Figure 7: Nucleotide sequence of the vit-2 promoter-NLS as present plasmid 20 pPM143
- Figure 8: Schematic drawing of pGN156
- Figure 9: Schematic drawing of pGQ1
- Figure 10: Schematic drawing of pGQ2
- Figure 11: Schematic drawing of pCLUC6
- 25 - Figure 12: Schematic drawing of pGQ3
- Figure 13: Schematic drawing of pGQ4
- Figure 14 : Stage specific expression of LacZ (C. elegans harboring pGN156) after one hour of probe addition.
- Figure 15 : Stage specific expression of LacZ (C. elegans harboring pGN156) after 30 two hours of probe addition.
- Figure 16: Stage specific expression of LacZ (C. elegans harboring pGN156) after three hours of probe addition.
- Figure 17: Expression of LacZ in function of the number of nematodes (C. elegans harboring pGN156).

- Figure 18: Fluorescence activity of adult nematodes (*C. elegans* UG1513) in flat bottom wells in function of the number of wells
- Figure 19 : Fluorescence activity of adult nematodes (*C. elegans* UG1513) in U-Shaped wells in function of the number of wells

5

Strain *C. elegans* UG1353 (pGN156) is deposited under accession number: "LMBP 5719CB", at the Belgian Coordinated Collection of Microorganisms (BCCM), Laboratorium voor moleculaire Biology-plasmidencollectie (LMBP) University of Ghent, K.L. Ledeganckstaat 35, 9000 Ghent; Belgium, according to the Budapest treaty of 28 April 1977 on the international recognition of the deposit of microorganisms for the purpose of patent procedures.

10

Examples:

15 Example 1: Construction of plasmids which allow for the expression of markers in a specific growth stage.

1) Construction of pGQ1 (ctl-1::GFP vector) (Figure 1, 9)

20 PCR was performed on genomic DNA isolated from *C. elegans* wild-type strain N2 under standard conditions with following primers:

oGQ1:

5'AAAACCTGCAGCCAATGCATTGGAAGAGATATTTGCGCGTCAAATATGTTTGTGT

CC3'

25 oGQ2:

5'CGCGGATCCGGCCGATTCTCCAGCGACCG3'

The PCR fragment was isolated and cloned as a PstI/BamHI fragment in pDW2020, resulting in pGQ1.

30 2) Construction of pGQ2 (ctl-2::luciferase vector) (Figure 3, 10)

PCR was performed on genomic DNA isolated from *C. elegans* wild-type strain N2 under standard conditions with following primers:

oGQ3:

5'CCAGGCCTGAGATATTTGCGCGTCAAATATGTTTGTGCC3'

oGQ4:

5'CGGAGCTCCGATTGGATGTGGTGAGCAGG3'

The PCR fragment was isolated and cloned as a StuI/SacI fragment in pCluc6, resulting

5 in pGQ2.

3) Construction of pGQ3 (sod-3::GFP vector) (figure 5, 12)

PCR was performed on genomic DNA isolated from *C. elegans* wild-type strain N2 under

10 standard conditions with following primers:

oGQ7: 5'GCAGAATTGCAAAACGAGCAGGAAAGTC3'

oGQ6: 5'TTGGCGCGCCAAGCCTTAATAGTGTCCATCAGC3'

The PCR fragment was isolated and cloned as a PstI/Ascl fragment in pDW2020, resulting in pGQ3.

15

4) Construction of pGQ4 (sod-3::luciferase vector) (Figure 6, 13)

PCR was performed on genomic DNA isolated from *C. elegans* wild-type strain N2 under standard conditions with following primers:

20 oGQ7: 5'GCAGAATTGCAAAACGAGCAGGAAAGTC3'

oGQ8: 5'CTGAGCTCGGCTTAATAGTGTCCATCAGC3'

The PCR fragment was isolated and cloned as a PstI/CacII fragment in pCluc6, resulting in pGQ4.

25

5) Construction of pCluc6 (vit-2::Luciferase vector) (Figure 2, 11)

PCR was performed on genomic DNA isolated from *C. elegans* wild-type strain N2 under standard conditions with following primers:

30 vit-2F: 5'CCCCCAAGCTTCCATGTGCTAGCTGAGTTCATCATGTCC3'

vit-2R: 5'CCCCCCAAGCTTGGCTGAACCGTGATTGG3'

The PCR fragment was isolated and cloned as a HindIII fragment in pCluc2, resulting in pCluc6.

6) Construction pGN156 (vit-2::lacZ vector) (Figure 4, 8)

The LacZ fragment of pPD95.4 (Fire et al, Gene Gene. 1990 Sep 14;93(2):189-98) was isolated as a Sful/Spel fragment and cloned in pPM143 (MacMorris et al., Gene expression vol. 3 no. p27, 1993) digested with the same enzymes, resulting in vector pGN156.

Example 2: Construction of *C. elegans* nematodes harboring the plasmids described above, and construction of stable integrated lines.

Each of the vectors was injected into *C. elegans* nematode worms using standard techniques as described in one of the references above. All the constructed transgenic strains showed the desired marker gene expression pattern, in a heritable way. Stable integrated line were constructed, an example is given for the integration of pGN156 (vit-2::lacZ):

- 1) *C. elegans* wild-type N2 nematodes have been injected with various concentrations of pGN156, reference and selection plasmid pGR6 (myo2::GFP), and carrier DNA (pUC18)
- 2) A good heritable strain was selected from the injection with 25ng pGN156, 5ng pGR6, 80 ng pUC18. Approximately 60 animals were gamm-irradiated (3000rad; 16x16 cm², 50 cm, 82.2 min) after which each worm was placed on a single plate and allowed to growth for offspring growth. Approximately 560 F1 offspring worms expressing GFP were placed each on a single plate, and allowed to grow. From The F2 generation, worms were again placed on single plates, and finally the F3 generation was checked for its GFP expression. The strains were then out-crossed with wild-type strain N2 to eliminate undesirable mutations, and checked for LacZ expression.
- 3) 6 selected nematodes, wherein pGN156 is integrated, were grown. From each culture, 10 nematodes were placed in the well of a 96 well plate, 25 µl M9 buffer (see above), 25 µl 60% ice cold Methanol, and 50 µl 20mM C12FDG probe(molecular probes) was added, the wells were further incubated for 2h at 37°C and

fluorescence was measured in a plate reader with following settings: ex/em:
485nm/535nm

4) One of the six strains showed high viability, strong GFP expression and relatively high LacZ expression and was selected for further analysis

5

This strain, designated *C. elegans* UG1353 (pGN156) is deposited under accession number: "LMBP 5719CB", at the Belgian Coordinated Collection of Microorganisms (BCCM), Laboratorium voor molecular Biology-plasmidencollectie (LMBP) University of Ghent, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium, according to the Budapest treaty of 28 April 1977 on the international recognition of the deposit of microorganisms for the purpose of patent procedures.

Example 3: LacZ-staining of an increasing number of *C. elegans* UG1353 (pGN156)

15 Transgenic nematodes, in various quantities per well, were dispensed using a worm dispenser: Copas 250NF (UBI), and the volume was added up to 35µL with M9 buffer. 35 µL C12FDG (molecular probes) and 35 µL 45% methanol was added. The wells were further incubated for at least 1h at 37°C. Fluorescence was measured with a Wallac Victor2 plate reader at ex/em: 485 nm/535 nm.

20 As shown in figure 18 and figure 19, the expression pattern of the transgenic nematodes is stable, which is clear from the linear increase of fluorescence versus a linear increase of nematodes in the wells.

Example 4: LacZ staining of *C. elegans* harboring pGN156 at various growth stages.

25 The expression pattern in function of the growth stage was measured. *C. elegans* harboring pGN156 was grown at various growth stages. Approximately 35 nematodes at various growth stages were placed in the wells of a microtiter plate. Each well contains only nematodes at a defined growth stage, being L1, L2-L3, L4, young adults, adults and older adults. M9 medium is added to a final volume of 35 µL.

30 35 µL 45% methanol and 35 µL 60 µM probe is added. Two probes have been tested:

- 1) Fluorescein di-beta-D-galactopyranoside (FDG) (Molecular Probes)
- 2) ImaGene green TM C12FDG (FDG) (Molecular Probes)

The probe was incubated for different time intervals (1 to 5 hours) at 37°C, after which the plates are cooled down to 30°C prior to measurement.

Measurement of fluorescence was performed described above. The results are shown in figures 14, 15, and 16, and clearly show that the marker gene under the control of the
5 vit-2 promoter is only expressed at the adult growth stage.

Further more linear relationship has also been tested between the number of worms added to the well and the fluorescence measured. Essential this has been performed in the same way as described above. Figure 17 shows the results, and the clear linearity
10 between the number of nematodes and the fluorescence.

Example 5: Constructing mutant strains harboring the integrates pGN156

The integrated pGN156 in *C. elegans* UG1353 can be crossed in any desired mutant
15 available (as provided by the references above, or by the CGC, university of Minnesota, St.-Paul), or in any mutant newly created. As an example the integrated line has been crossed in a Daf-2 mutant line.

Strain UG1353 was crosses with a Wild-type male (N2) resulting in heterozygote males
20 and hermaphrodites. A daf-2 (m41) strain was crossed with the herterozygote strain isolated above. From the offspring, the GFP expressing nematodes were isolated, and allowed self-fertilization, once again, L4 stage nematodes were isolated which express GFP, and the nematodes were placed at 25°C to allow o form dauers. Dauers were isolated and further incubated at 15°C. The offspring was analysed and nematodes
25 which have a 100% GFP expressing offspring are isolated for further analysis. These analysed nematodes are homozygote for both the integration of PGN156 and for daf-2 (mp41)

Example 6: Screening for compounds that affect dauer formation using the daf-2 (PNZ156) nematodes of the example above.

The *C. elegans* daf-2 (pNZ156) nematodes were synchronized, and approximately 50 nematodes at the L1 stage were placed in each well of a 96 well plate. S medium was added as well as *E. coli* as described above to a final volume of 50 µL. Compound was

added at a final concentration of 30 µL and the nematodes were allowed to grow between 22°C and 25°C for approximately 4 days, dependent on the temperature chosen.

Methanol and probe was than added as described in the examples above to allow the 5 detection of the expression of the LacZ marker, and the wells were further incubated for 1 hour to overnight as described above, after which the fluorescence was measured, as described above.

At a temperature higher than 22°C this strains enters the dauer stage, at which stage no vit-2 expression, and hence no LacZ expression can be observed. Compounds which 10 allow the nematode to bypass the dauer stage, and hence allows the nematodes to growth till the adult stage, will result in the expression of lacZ. Hence, fluorescence is detected in the wells where nematodes have been grown till at least the adult stage, hereby selecting a compound that affect dauer formation.

15 Example 7: selection of synchronized worms

Example 8: selection of mutants

Chemical mutagenesis has been described extensively in *C. elegans*, Modern biological 20 analysis of an organism, Methods in Cell Biology, Vol 48. Transposon mutagenesis has been described in WO 00/73510 (PCT/US00/40091). In general, the desired mutated nematodes are selected which have a desired phenotype by microscopy. When these mutagenesis techniques are performed with transgenic strains harboring a marker gene such as GFP under the control of a growth stage specific promoter, this allows for a 25 faster and automated selection.

In Short:

Approximately 1000000 eggs of a strain harboring a marker gene under the regulation of a growth stage dependent promoter (such as vit-2::GFP) are grown till L4-young adult stage after which they are treated with the mutagen. They are allowed to growth further 30 on plates (approximately 25.000 worms per plate). The nematodes are washed off the plates with M9 buffer, while the eggs (harboring the mutants) are allowed to grow further. The L1 offspring is then washed off and filtered using a 20µM nylon membrane (millipore).

The L1 nematodes (F1) contain the desired dominant mutants. Depending on the desired phenotype, between 2 and 50 worms are then placed in the wells of a 96 well plate, and allowed to grow further. The plates are place into a plate reader at various time intervals (approximately every 12 hours) to check the growth speed. As mutants are known to have a slower growth speed, selection can be made automatically the mutants that grow slower or selected for further analysis.

To select for recessive mutants, the L1 nematodes (F1) are allowed to grow further, and the resulting young adults are placed (approximately 500 per plate) on plates.

The eggs are isolated as above and allow to grow further till L1 stage (F2) prior to the dispensing of the nematodes into the wells, as described above. The selection occurs as described for the F1 generation.

Example 9: Selection in resistance genetics

A particular kind of mutants to be selected, are those mutants who show resistance to a compound. The addition of an active compound to a nematode result mainly in growth delay, growth arrest, lethality, and/or paralysis. Analogous as in the assay described above, mutant nematodes can be isolated that are resistant to the compound. Such mutant can be selected as the mutants will overcome the induction of the phenotype induced by the compound, and hence growth faster than the none mutated nematodes.

The mutagenesis is performed as described above, while the assay and the outcome is different. In the well plates, were the L1 nematodes are allowed to grow, the compound is added. The concentration is dependent on the compound and may be between 10 µM and 350 µM, preferably 100 µM. As such compound resistance mutants will grow faster than the non-mutated nematodes, selection of the desired mutants occurs by selecting the nematodes that show firstly expression, which also has been done automatically.

Example 10: Growth monitoring in RNAi screens

Analogous to the mutagenesis methods above, dsRNA inhibition can be performed. The principle of HTS RNAi has bee described in WO 00/01846, Nematodes can be feed by bacteria that express high amounts of dsRNA. Such RNA crossed the gut barrier, and enters the cells of *C. elegans* performing is RNA inhibitory action.

In short:

Approximately 3 to 5 L1 synchronized nematodes (harboring a marker gene under the regulation of stage specific promoter) are placed in the wells of a microtiter plate, in

which also *E. coli* bacteria are present that express high levels of dsRNA. The nematodes are allowed to grow, and those are selected that show lethality, growth delay, growth arrest, etc, which can automatically be measured as these nematodes will not enter the growth stage that allows the expression of the marker gene. The assay to

5 select for the desired *E. coli* (harboring dsRNA expression of the gene of interest) is essential the same as the assay described above for mutagenesis.

In addition, a compound that induces growth delay, growth arrest, paralysis, or lethality can be added to the wells, at appropriate concentrations as described above. RNAi action on the nematode can induce resistance to such compound, analogous as has

10 been described above for compound resistance selection. Also in this case, the nematodes are selected that overcome the phenotype induced by the compound, as they will grow faster than the nematodes that have not acquired resistance by the RNAi. As the nematodes harbor a functional promoter marker fusion, such as vit-2::GFP, only the nematodes that grow (fast), will express the marker, and hence can be selected.

15

CLAIMS

1. Method for determining the influence of at least one exogenous factor on the development and/or growth of a sample of nematode worms, said method comprising:

- 5 a) providing a sample of nematode worms,
 in which said nematode worms contain a marker gene operably linked to a promoter,
 which promoter is capable of driving the expression of the marker gene in the nematode worms such that the marker gene is not expressed in at least a first development stage of the nematodes, but is expressed in at least a second development stage of the nematodes (different from the first life stage);
b) exposing said sample of nematode worms to at least one exogenous factor;
c) maintaining/cultivating said sample of nematode worms in a suitable medium,
15 optionally over one or more life stages and/or generations;
d) subjecting the sample of nematode worms to at least one detection technique that is capable of detecting the signal generated by the marker gene (if expressed).

2. Method for determining the influence of at least a first exogenous factor on the development and/or growth of a sample of nematode worms, said method comprising:

- 20 a) providing at least a first and a second sample of nematode worms,
 in which the nematode worms in each sample contain a marker gene operably linked to a promoter,
 which promoter is capable of driving the expression of the marker gene in the nematode worms such that the marker gene is not expressed in at least a first development stage of the nematodes, but is expressed in at least a second development stage of the nematodes (different from the first life stage);
b) exposing at least said first sample of nematode worms to said first one exogenous
30 factor;
c) maintaining/cultivating said samples of nematode worms in a suitable medium,
 optionally over one or more life stages and/or generations;
d) subjecting the samples of nematode worms to at least one detection technique that is capable of detecting the signal generated by the marker gene (if expressed);

e) determining the time required for the first sample of nematode worms to show expression of the marker gene (as determined by the signal detected for the first sample in step b)), and preferably also determining the time required for the second sample of nematode worms to show expression of the marker gene (as determined by the signal detected for the second sample in step b)); and/or comparing the time required for the first sample of nematode worms to show expression of the marker gene with the time required for the second sample of nematode worms to show expression of the marker gene.

10 3. Method according to claim 2, in which the second sample of nematode worms is not exposed to any exogenous factor.

4. Method according to claim 2, in which the second sample of nematode worms is exposed to a second exogenous factor.

15

5. Method according to any of the preceding claims, in which nematodes used are preferably from the genus *Caenorhabditis*, such as from *Caenorhabditis briggsae* or *Caenorhabditis elegans*.

20

6. Method according to claim any of the preceding claims, in which the first development stage is chosen from eggs, an embryonal stage, L1, L2 and dauer.

7. Method according to claim any of the preceding claims, in which the first development stage is L1.

25

8. Method according to claim any of the preceding claims, in which the first development stage is chosen from L4, adult or dauer.

30

9. Method according to any of the preceding claims, in which the promoter chosen from any one of the following promoters: gpl-1, unc-54, myo-2, lin-28, lin-4, lin-14, col-7, col-19, col-17, ctl-1, sod-3, vit-2.

10. Method according to any of the preceding claims, in which the promoter is the vit-2 promoter.

11. Method according to any of the preceding claims, in which marker gene is chosen from green fluorescent protein, beta-galactosidase, beta-lactamase, luciferase, acetohydroxyacid synthase, alkaline phosphatase, beta-glucuronidse, chloramphenicol acetyltransferase, horseradish peroxidase, nopaline synthase and/or octapine synthase.

5

12. Method according to any of the preceding claims, in which marker gene encodes a gene product that is toxic (e.g. lethal) to the nematode.

10 13. Method according to any of the preceding claims, in which step d) is carried out using a non-visual detection technique.

14. Method according to any of the preceding claims, which is carried out in multi-well plate format.

15 15. Method according to any of the preceding claims, which is carried out in an automated fashion.

16. Method according to any of the preceding claims, in which the at least one exogenous factor is at least one small compound or at least one small peptide.

20

17. Method according to any of the preceding claims, in which the at least one exogenous factor is a double stranded RNA sequence, suitable or intended for suppression the expression of at least one nucleotide sequence in the nematode worm by means of RNA interference.

25

18. Method according to any of the preceding claims, in which the nematode worms have been subjected to mutagenesis prior to use in step a).

30 19. Use of a (sample of at least one) nematode worm, which nematode worm contains a marker gene operably linked to a promoter, which promoter is capable of driving the expression of the marker gene in the nematode worms such that the marker gene is not expressed in at least a first development stage of the nematodes, but is expressed in at least a second development stage of the nematodes (different from the

first life stage), in a method or assay for determining the influence of at least one exogenous factor on the development and/or growth on a nematode worm.

20. Use according to claim 19, in which nematodes used are preferably from the
5 genus *Caenorhabditis*, such as from *Caenorhabditis briggsae* or *Caenorhabditis elegans*.

21. Use according to claim 19 or 20, in which the promoter chosen from any one
of the following promoters: gpl-1, unc-54, myo-2, lin-28, lin-4, lin-14, col-7, col-19, col-17,
10 ctl-1, sod-3, vit-2.

22. Use according to any of claims 19-21, in which the promoter is the vit-2
promoter.

15 23. Use according to any of claims 19-22, in which the marker gene is chosen
from green fluorescent protein, beta-galactosidase, beta-lactamase, luciferase,
acetohydroxyacid synthase, alkaline phosphatase, beta-glucuronidase, chloramphenicol
acetyltransferase, horseradish peroxidase, nopaline synthase and/or octapine synthase.

20 24. Use according to any of claims 19-22, in which the marker gene encodes a
gene product that is toxic (e.g. lethal) to the nematode.

*1/27**FIG. 1.* Nucleotide sequence of pGQ1

ATGACCATGA TTACGCCAAG CTTGCATGCC TGCAGCCAAT GCATTGGAAG
AGATATTTG CGCGTCAAAT ATGTTTGTC TCCCCGTAAT ATTTTTAA
ATCAAATTTC ACATTTAAC CATAAAAAC TCTTCAAAA GTGTAATTT
CTACGCAAAA ATGCCGTTCG GATGAAAAAT TACTTTGAA AAACAAACTC
GAAACTACGG TACGCCAAA AGTACATCGG TGTTTGCACA TAAGTGA
CAATGTTGTT TTTTGTAAT TAAAATCGAT TAATTTTTT TCCCGGAAAA
CAAAACGTT TTCAGCGTGG ATTCTTATTG TTTCTGCGT AAAAAAAAT
TATTTACCAA TTTTAAACGA TAATTCAC GAATTTCGC CATTAATCTC
TCGATTTGT TGATTCTGA CTCCGAGCAA TCTCTCCGGT TTTCGCAAAC
GATTATATTA TTTATTTGTT TTCCCTTTCA GTGCCGATT TC GGAAATT
AACAGTAAAT CTTCAAAATG CCAATGCTTC CCCACATGGT CAATCTAA
GAGTTCTTT GTTACAAAAT ACACGTGATG TCAGATTGTC TCATTCGGT
TTGATCTACG TAGATCTACA AAAAATGCGG GAATTGAGCC GCAGAGTTCT
CAACTGCTTT CGCATGGTTA AGAACGTGCG GACGTCAAAT TGTTTGGC
AAAAAATTCCC GCATTTTTG TAGATCAAAC CGTAATGGGA CAGTCGGCA
CCACGTGACT ATATATTTC AGCGGTCAAC GACACAAAAC CGGGACCAAT
GGCTGAGGAT CAGCTGAAAG CTTATAGAGA TAGAAATCAG GTGAGAAAAA
TCAATTCAG CGATTTCTT CGCAATTAT ATAAAAACTG ATTTTCCAG
GAACCCCCACC TGCTCACAC ATCCAATGGG GCTCCGATCT ACTCGAAGAC
CGCCGTGTC ACCGCCGGAC GACGGTGGTCC AATGCTAATG CAGGACATCG
TTTATATGGA CGAGATGGT CATTTCGATC GTGAACGCAT CCCGGAGCGT
GTCGTCCATG CCAAAGGGTGG TGGTGTCTAT GGATACTTCG AGGTCA
TGACATCACC AAGTACTGTA AGGGCGATAT GTTCAACAAG GTCGGAAA
AGACACCACT TCTCGTTCGT TTTCAACGG TCGCTGGAGA ATCGGGCGGA
TCCCCGGGAT TGGCCAAAGG ACCCAAAGGT ATGTTTCGAA TGATA
ATAACATAGA ACATTTTCAG GAGGACCTT GGCTAGCGTC GACGGTACCA
TGGGGCGCGC CATGAGTAA GGAGAAGAAC TTTTCACTGG AGTTGCCCC
ATTCTGTTG AATTAGATGG TGATGTTAAT GGGCACAAAT TTTCTGTCAG
TGGAGAGGGT GAAGGGTGTG CAACATACGG AAAACTTAC CTTAAATT
TTTGCACTAC TGGAAAACA CCTGTTCCAT GGGTAAGTTT AACATATAT
ATACTAACTA ACCCTGATTA TTTAAATTTC CAGCCAACAC TTGTC
TTTCTGTTAT GGTGTTCAAT GCTTCTCGAG ATACCCAGAT CATAGAAC
GGCATGACTT TTTCAAGAGT GCCATGCCCG AAGGTTATGT ACAGGAAAGA
ACTATATTT TCAAAGATGA CGGGAACTAC AAGACACGTA AGTTAAACA
GTCGGTACT AACTAACCAT ACATATTAA ATTTTCAGGT GCTGAAGTC
AGTTTGAAGG TGATACCCCTT GTTAATAGAA TCGAGTTAAA AGGTATTGAT
TTTAAAGAAG ATGAAACAT TCTTGGACAC AAATTGGAAT ACAACTATA
CTCACACAAT GTATAACATCA TGGCAGACAA ACAAAAGAAT GGAATCAA
TTGTAAGTTT AAACCTGGAC TTACTAACTA ACGGATTATA TTTAAATT
CAGAACTTCA AAATTAGACA CAACATTGAA GATGGAAGCG TTCAACTAGC
AGACCATTAT CAACAAAATA CTCCAATTGG CGATGGCCCT GTCC
CAGACAACCA TTACCTGTCC ACACAATCTG CCCTTCGAA AGATCC
GAAAAGAGAG ACCACATGGT CCTCTTGTAG TTTGTAACAG CTGCTGGGAT
TACACATGGC ATGGATGAAC TATACAAATA GGGCCGGCCG AGCTCC
CGGCCGCTGT CATCAGATCG CCATCTCGCG CCCGTGCTCTC TGACTT
GTCCAATTAC TCTTCAACAT CCCTACATGC TCTTCTCCC TGTGCT
CCCCCTATT TTGTTATTAT CAAAAAAACT TCTTCTTAAT TTCTTGT
TTTAGCTCT TTTAAGTCAC CTCTAACAAAT GAAATTGTGT AGATCA
ATAGAATTAA TTCGTAATAA AAAGTCGAA AAAATTGTGC TCCCTCCCCC

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FIG. 1 (CONTINUED 1).

CATTATAAT AATTCTATCC CAAAATCTAC ACAATGTTCT GTGTACACTT
 CTTATGTTT TTTACTTCT GATAAATTT TTTGAAACA TCATAGAAAA
 AACCGCACAC AAAATACCT ATCATATGTT ACGBTTCAGT TTATGACCGC
 AATTTTATT TCTTCGCACG TCTGGGCCTC TCATGACGTC AAATCATGCT
 CATCGTAAA AAGTTTGA GTATTTTGG AATTTTCAA TCAAGTGAAA
 GTTTATGAAA TTAATTTCC TGCTTTGCT TTTGGGGGT TTCCCCTATT
 GTTTGTCAG AGTTTCGAGG ACGGCGTTT TCTTGCTAAA ATCACAAGTA
 TTGATGAGCA CGATGCAAGA AAGATCGAA GAAGGTTGG GTTTGAGGCT
 CAGTGGAGG TGAGTAGAAG TTGATAATT GAAAGTGGAG TAGTGTCTAT
 GGGGTTTTG CCTTAAATGA CAGAATACAT TCCCAATATA CCAAACATAA
 CTGTTTCTA CTAGTCGGCC GTACGGGGCC TTTCGTCTCG CGCGTTTCGG
 TGATGACGGT GAAAACCTCT GACACATGCA GCTCCCGGAG ACGGTCACAG
 CTTGTCGTGTA AGCGGATGCC GGGAGCAGAC AAGCCCGTCA GGGCGCTCA
 GCGGGTGTG GCGGGTGTGCG GGGCTGGCTT AACTATGCGG CATCAGAGCA
 GATTGTACTG AGAGTGCACC ATATGCGGTG TGAATACCG CACAGATGCG
 TAAGGAGAAA ATACCGCATC AGGCGGCCTT AAGGGCCTCG TGATACGCCT
 ATTTTATAG GTTAATGTC TGATAATAAT GGTTCTTAG ACGTCAGGTG
 GCACCTTCTG GGGAAATGTG CGCGGAACCC CTATTGTTT ATTTTCTAA
 ATACATCAA ATATGATCC GCTCATGAGA CAATAACCC GATAAATGCT
 TCAATAATAT TGAAAAGGA AGAGTATGAG TATTCAACAT TTCCGTGTCG
 CCCTTATTCC CTTTTTGC GCACTTGC TTCCGTGTT TGCTCACCCA
 GAAACGCTGG TGAAAGTAAA AGATGCTGAA GATCAGTTGG GTGCACGAGT
 GGGTTACATC GAACTGGATC TCAACAGCGG TAAGATCCTT GAGAGTTTC
 GCCCCGAAGA ACGTTTCCA ATGATGAGCA CTTTAAAGT TCTGCTATGT
 GGCGCGGTAT TATCCGTAT TGACGCCGG CAAGAGCAAC TCGGTCGCCG
 CATAACTAT TCTCAGAATG ACTTGGTGA GTACTCACCA GTCACAGAAA
 AGCATCTTAC GGATGGCATG ACAGTAAGAG ATTATGCAAG TGCTGCCATA
 ACCATGAGTG ATAACACTGC GGCCAACCTA CTTCTGACAA CGATCGGAGG
 ACCGAAGGAG CTAACCGCTT TTTGCACAA CATGGGGAT CATGTAACCTC
 GCCTTGATCG TTGGGAACCG GAGCTGAATG AAGCCATACC AAACGACGAG
 CGTGACACCA CGATGCCGT AGCAATGGCA ACAACGTTGC GCAAACATT
 AACTGGCGAA CTACTTACTC TAGCTTCCCG GCAACAATTA ATAGACTGGA
 TGGAGGGGGA TAAAGTTGCA GGACCACCTC TCGCCTCGGC CCTTCCGGCT
 GGCTGGTTA TTGCTGATAA ATCTGGAGCC GGTGAGCGTG GGTCTCGCGG
 TATCATTGCA GCACTGGGGC CAGATGGTAA GCCCTCCCGT ATCGTAGTTA
 TCTACACGAC GGGGAGTCAG GCAACTATGG ATGAACGAAA TAGACAGATC
 GCTGAGATAG GTGCCCTACT GATTAAGCAT TGGTAACTGT CAGACCAAGT
 TTACTCATAT ATACTTTAGA TTGATTTAAA ACTTCATT TTAAATTTAAA
 GGATCTAGGT GAAGATCCTT TTTGATAATC TCATGACCAA AATCCCTTAA
 CGTGAGTTT CGTTCCACTG AGCGTCAGAC CCCGTAGAAA AGATCAAAGG
 ATCTTCTGA GATCCTTTT TTCTGCCGT AATCTGCTGC TTGCAAACAA
 AAAAACCAAC GCTACCAGCG GTGGTTTGTG TGCCGGATCA AGAGCTACCA
 ACTCTTTTC CGAAGGTAAC TGGCTTCAGC AGAGCGCAGA TACCAAATAC
 TGTCTTCTA GTGTAGCCGT AGTTAGGCCA CCACCTCAAG AACTCTGTAG
 CACCGCCTAC ATACCTCGCT CTGCTAATCC TGTTACCAAGT GGCTGCTGCC
 AGTGGCGATA AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC
 GGATAAGGCG CAGCGGTGCG GCTGAACGGG GGGTCGTGC ACACAGCCCA
 GCTTGGAGCG AACGACCTAC ACCGAACGTGA GATAACCTACA GCGTGAGCAT
 TGAGAAAGCG CCACGCTTCC CGAAGGGAGA AAGGCGGACA GGTATCCGGT
 AAGCGGCAGG GTCGGAACAG GAGAGCGCAC GAGGGAGCTT CCAGGGGAA
 ACGCCTGGTA TCTTATAGT CCTGTCGGGT TTGGCCACCT CTGACTTGAG

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FIG. 1 (CONTINUED 2).

CGTCGATTT TGTGATGCTC GTCAGGGGG CGGAGCCTAT GGAAAAACGC
CAGCAACGCG GCCTTTTAC GGTTCTGGC CTTTGCTGG CCTTTTGCTC
ACATGTTCTT TCTTGCCTTA TCCCCTGATT CTGTGGATAA CCGTATTACC
GCCTTGAGT GAGCTGATAC CGCTGCCGC AGCCGAACGA CCGAGCGCAG
CGAGTCAGTG AGCGAGGAAG CGGAAGAGCG CCCAATACGC AAACCGCCTC
TCCCCCGCGCG TTGGCCGATT CATTAATGCA GCTGGCACGA CAGGTTTCCC
GACTGGAAAG CGGGCAGTGA GCGCAACGCA ATTAATGTGA GTTAGCTCAC
TCATTAGGCA CCCCAGGCTT TACACTTTAT GCTTCCGGCT CGTATGTTGT
GTGGAATTGT GAGCGGATAA CAATTCACA CAGGAAACAG CT

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FIG. 2. Nucleotide sequence of PCLUC6

ATGACTGCTC CAAAGAAGAA GCGTAAGGTA CCGGTAGAAA AAATGGAAGA
CGCCAAAAAC ATAAAGAAG GCCCGGCGCC ATTCTATCCG CTGGAAGATG
GAACCGCTGG AGAGCAACTG CATAAGGCTA TGAAGAGATA CGCCCTGGTT
CCTGGAACAA TTGCTTTAC AGATGCACAT ATCGAGGTGG ACATCACTTA
CGCTGAGTAC TTCGAAATGT CCGTTCGGTT GGCAGAGCT ATGAAACGAT
ATGGGCTGAA TACAAATCAC AGAACATCG TATGCAGTGA AAAACTCTT
CAATTCTTTA TGCCGGTGTGTT GGGCGCGTTA TTATCGGAG TTGCAGTTGC
GCCCGCGAAC GACATTTATA ATGAACGTGA ATTGCTCAAC AGTATGGGCA
TTTCGAGGCC TACCGTGGTG TTCGTTCCA AAAAGGGGTT GCAAAAAATT
TTGAACGTGC AAAAAAAAGCT CCCAACATC CAAAAAAATTA TTATCATGG
TTCTAAAACG GATTACCAGG GATTTCAGTC GATGTACACG TTCGTACAT
CTCATCTACC TCCCAGGTTT AATGAATAACG ATTGCTGTGCC AGAGTCCTC
GATAGGGACA AGACAATTGC ACTGATCATG AACTCCTCTG GATCTACTGG
TCTGCCCTAA GGTGTCGCTC TGCCTCATAG AACTGCCTGC GTGAGATTCT
CGCATGCCAG AGATCCTATT TTTGCAATC AAATCATTCC GGATACTGCG
ATTTAAGTG TTGTTCCATT CCATCACGGT TTGGAATGT TTACTACACT
CGGATATTG ATATGTGGAT TTCGAGTCGT CTTAATGTAT AGATTTGAAG
AAGAGCTGTT TCTGAGGAGC CTTCAAGGATT ACAAGATTCA AAGTGCCTG
CTGGTGCCAA CCCTATTCTC CTTCTCGCC AAAAGCACTC TGATTGACAA
ATACGATTAA TCTAATTAC ACGAAATTGC TTCTGGTGGC GCTCCCCCTCT
CTAAGGAAGT CGGGGAAGCG GTTCCAAGA GGTTCCATCT GCCAGGTATC
AGGCAAGGAT ATGGGCTCAC TGAGACTACA TCAGCTATT TGATTACACC
CGAGGGGGAT GATAAACCGG GCGCGGTGCG TAAAGTTGTT CCATTTTTG
AAGCGAAGGT TGTGGATCTG GATACCGGGA AACAGCTGG CGTTAACCAA
AGAGGCGAAC TGTGTGTGAG AGGTCTATG ATTATGTCCG GTTATGTAAA
CAATCCGGAA GCGACCAACG CCTTGATTGA CAAGGATGGA TGGCTACATT
CTGGAGACAT AGCTTACTGG GACGAAGACG AACACTTCTT CATCGTTGAC
CGCCTGAAGT CTCTGATTAA GTACAAAGGC TATCAGGTGG CTCCCGCTGA
ATTGGAATCC ATCTTGCTCC AACACCCCAA CATCTTCGAC GCAGGTGTCG
CAGGTCTTCC CGACGATGAC GCGGTGAAC TTCCCGCCGC CGTTGTTGTT
TTGGAGCAG GAAAGACGAT GACGGAAAAA GAGATCGTGG ATTACGTCGC
CAGTCAAGTA ACAACCGCGA AAAAGTTGCG CGGAGGAGTT GTGTTTGTGG
ACGAAGTACC GAAAGGTCTT ACCGGAAAAC TCGACGCAAG AAAAATCAGA
GAGATCCTCA TAAAGGCCAA GAAGGGCGGA AAGATCGCCG TGTAAATTCTA
GGAATTCCAA CTGAGCGCCG GTCGCTACCA TTACCAACTT GTCTGGTGT
AAAAATAATA GGGGCGCTG TCATCAGAGT AAGTTAAC TGAGTTCTAC
TAACTAACGA GTAATATTAA AATTTCAGC ATCTCGCGCC CGTGCCTCTG
ACTTCTAAGT CCAATTACTC TTCAACATCC CTACATGCTC TTTCTCCCTG
TGCTCCCACC CCCTATTGTT GTTATTATCA AAAAAGCTTC TTCTTAATT
CTTTGTTTT TAGCTTCTT TAAGTCACCT CTAAACAATGA AATTGTGTAG
ATTCAAAAT AGAATTAATT CGTAATAAAA AGTCGAAAAA AATTGTGCTC
CCTCCCCCCTA TTAATAATAA TTCTATCCCA AAATCTACAC AATGTTCTGT
GTACACTTCT TATGTTTT TTACTCTGA TAAATTTTT TTGAAACATC
ATAGAAAAAA CCGCACACAA AATACCTTAT CATATGTTAC GTTTCAGTTT
ATGACCGCAA TTTTATTTC TTGCGACGTC TGGGCCTCTC ATGACGTCAA
ATCATGCTCA TCGTAAAAA GTTTGGAGT ATTTTTGGAA TTTTCAATC
AAGTGAAGT TTATGAAATT AATTTCCTG CTTTTGCTTT TTGGGGGTTT
CCCTATTGT TTGTCAGAGGAG TTTGAGGAC GGCCTTTTC TTGCTAAAAT
CACAAGTATT GATGAGCACG ATGCAAGAAA GATCGGAAGA AGGTTGGGT

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FIG. 2 (CONTINUED 1).

TTGAGGCTCA GTGGAAGGTG AGTAGAAGTT GATAATTGA AAGTGGAGTA
GTGTCTATGG GGTTTTGCCC TTAAATGACA GAATACATTC CCAATATACC
AACACATAACT GTTTCCTACT AGTCGGCGT ACGGGCCCTT TCGTCTCGCG
CGTTTCGGTG ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCGGAGAC
GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCGCGTCAGG
GCGCGTCAGC GGGTGTGGC GGGTGTGGG GCTGGCTTAA CTATGCGGCA
TCAGAGCAGA TTGTACTGAG AGTCACCAT ATGCGGTGTG AAATACCGCA
CAGATGCGTA AGGAGAAAAT ACCGCATCAG GCGGCCTTAA GGGCCTCGTG
ATAGGCCTAT TTTTATAGGT TAATGTCATG ATAATAATGG TTTCTTAGAC
GTCAGGTGGC ACTTTTCGGG GAAATGTGCG CGAAACCCCT ATTTGTTTAT
TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACAA ATAACCCCTGA
TAAATGCTTC ATAATATTTG AAAAGGAAAG AGTATGAGTA TTCAACATTT
CCGTGTCGCC CTTATTCCCT TTTTGCAGC ATTTGCCTT CCTGTTTTG
CTCACCCAGA AACGCTGGTG AAAGTAAAAG ATGCTGAAGA TCAGTTGGGT
GCACCGAGTGG GTTACATCGA ACTGGATCTC AACAGCGGTAA AGATCCTTGA
GAGTTTCGC CCCGAGAAC GTTTTCCAAT GATGAGCACT TTTAAAGTTC
TGCTATGTGG CGCGGTATTA TCCCGTATTG ACGCCGGGCA AGAGCAACTC
GGTCGCGCAGA TACACTATTC TCAGAATGAC TTGGTTGAGT ACTCACCAGT
CACAGAAAAG CATCTTACGG ATGGCATGAC AGTAAGAGAA TTATGCGATG
CTGCCATAAC CATGAGTGT AACACTGCG CCAACTTACT TCTGACAACG
ATCGGAGGAC CGAAGGAGCT AACCGCTTT TTGACACAACA TGGGGGATCA
TGTAACTCGC CTTGATCGT GGGAACCGGA GCTGAATGAA GCCATACCAA
ACGACGAGCG TGACACCAAG ATGCCCTGTAG CAATGGCAAC AACGTTGCGC
AAACTATTAA CTGGCGAAC ACTTACTCTA GCTTCCCGGC AACAAATTAAAT
AGACTGGATG GAGGCGGATA AAGTTGCAGG ACCACTCTG CGCTCGGCC
TTCCGGCTGG CTGGTTTATT GCTGATAAAAT CTGGAGCCGG TGAGCGTGGG
TCTCGCGGTAA TCATTGCAAGC ACTGGGGCCA GATGGTAAGC CCTCCCGTAT
CGTAGTTATC TACACGACGG GGAGTCAGGC AACTATGGAT GAACGAAATA
GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG GTAAGTGTCA
GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTA
ATTAAAAGG ATCTAGGTGA AGATCCTTT TGATAATCTC ATGACCAAAA
TCCCTTAACG TGAGTTTCG TTCCACTGAG CGTCAGACCC CGTAGAAAAG
ATCAAAGGAT CTTCTTGAGA TCCTTTTTT CTGCGCGTAA TCTGCTGTT
GCAAACAAAA AAACCAACCGC TACCAAGCGGT GGTTGTTTG CCGGATCAAG
AGCTACCAAC TCTTTTTCCG AAGGTAACTG GCTTCAGCAG AGCGCAGATA
CCAAATACTG TCCCTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAGAA
CTCTGTAGCA CCGCCTACAT ACCTCGCTCT GCTAATCCTG TTACCATGTT
CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CGGGGTTGGA CTCAAGACGA
TAGTTACCGG ATAAGGCAGCA GCGGTGGGGC TGAACGGGGG GTTCTGAC
ACAGCCCAGC TTGGAGCGAA CGACCTACAC CGAACTGAGA TACCTACAGC
GTGAGCATTG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA GGCAGCTTCC
TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCAGGA GGGAGCTTCC
AGGGGGAAAC GCCTGGTATC TTATAGTCC TGTCGGGTT CGCCACCTCT
GACTTGAGCG TCGATTTTG TGATGCTCGT CAGGGGGGGC GAGCCTATGG
AAAACGCCA GCAACGCCG CTTTTACGG TTCTGGCCT TTTGCTGGCC
TTTGCTCAC ATGTTCTTC CTGCGTTATC CCCTGATTCT GTGGATAACC
GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCGCAG CGAACGACC
GAGCGCAGCG AGTCAGTGTAG CGAGGAAGCG GAAGAGCGCC CAATACGAA
ACCGCCTCTC CCCGCGCGTT GGCGATTCA TTAATGCAGC TGGCACGACA
GGTTTCCCGA CTGGAAAGCG GGCAGTGAGC GCAACGCAAT TAATGTGAGT
TAGCTCACTC ATTAGGCACC CCAGGTTTA CACTTTATGC TTCCGGCTCG

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FIG. 2 (CONTINUED 2).

TATGTTGTGT GGAATTGTGA CGGGATAACA ATTCACACA GGAAACAGCT
ATGACCATGA TTACGCCAAG CTGTAAGTTT AAACATGATC TTACTAACTA
ACTATTCTCA TTTAAATTCT CAGAGCTAA AAATGGCTGA AATCACTCAC
AACGATGGAT ACGCTAACAA CTTGGAAATG AAATAAGCTT GCATGCCTGC
AGGCCTTGGT CGACTCTAGA GGATCAAACCT GTATTACTTG AAACAATTAA
GTTATATGTT TAGAACCCCT CATTCAAAAT TAATAGACAG GGCTCTCACC
GAATGTTGCA ATTTGTTCT GATAAGGGTC ACAAAAGCGGA GCGAATGCTT
GAATGTTGCC ATCAATGAGC TTATCAATGC GCTAAAACGC TATAACTTCC
ATATGAAGTC AATCGAACAT ATGTCAATCT TTAGCCGTAT ATAAAGGTGC
ACTGAAAACA GTCCAATCAC GGTCAGCCA TGAGGTCGAT CCCCAGGCCGG
GATTGGCCAA AGGACCCAAA GGTATGTTTC GAATGATACT AACATAACAT
AGAACATTTT CAGGAGGACC CTTGGAGGGT ACCGGGGATT GGCAAAGGA
CCCAAAGGTA TGTTTCGAAT GATACTAACAA TAACATAGAA CATTTCAGG
AGGACCCCTTG CTTGGAGGGT ACCGAGCTCA GAAAAAA

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FIG. 3. Nucleotide sequence of pGQ2

ATGACTGCTC CAAAGAAGAA GCGTAAGGTA CCGGTAGAAA AAATGGAAGA
 CGCCAAAAAC ATAAGAAGAG GCCCCGGCGCC ATTCTATCCG CTGGAAGATG
 GAACCGCTGG AGAGCACTG CATAAGGCTA TGAAGAGATA CGCCCTGGTT
 CCTGGAACAA TTGCTTTAC AGATGCACAT ATCGAGGTGG ACATCACTTA
 CGCTGAGTAC TTCGAAATGT CCGTCGGTT GGCAGAAGCT ATGAAACGAT
 ATGGGCTGAA TACAATCAC AGAACATCG TATGCAGTGA AAACCTCTT
 CAATTCTTTA TGCCGGTGT GGGCGCGTT TTTATCGGAG TTGCAGTTGC
 GCCCGCGAAC GACATTATA ATGAACTGTA ATTGCTAAC AGTATGGCA
 TTTCGCAGCC TACCGTGGTG TTCGTTCCA AAAAGGGGTT GCAAAAAATT
 TTGAACGTGC AAAAAAGCT CCCAATCATC CAAAAAAATT TTATCATGGA
 TTCTAAACG GATTACCAAGG GATTTCACTG GATGTACACG TTGTCACAT
 TTCATCTACC TCCCGGTTT AATGAATACG ATTTGTGCC AGAGTCCTTC
 GATAGGGACA AGACAATTGC ACTGATCATG AACTCCCTG GATCTACTGG
 TCTGCCAAA GGTGTCGCTC TGCCCTCATAG AACTGCGCTG GTGAGATTCT
 CGCATGCCAG AGATCCTATT TTTGGCAATC AAATCATTCC GGAACTGCG
 ATTTAAGTG TTGTTCCATT CCATCACGGT TTGGAATGT TTACTACACT
 CGGATATTG ATATGTGGAT TTCGAGTCGT CTTAATGTAT AGATTTGAAG
 AAGAGCTTT TCTGAGGAGC CTTCACTG ACAAGATTCA AAGTGCCTG
 CTGGTCCAA CCCTATTCTC CTTCTCGCC AAAAGCACTC TGATTGACAA
 ATACGATTAA TCTAATTAC ACGAAATTGC TTCTGGTGGC GCTCCCCCT
 CTAAGGAAGT CGGGGAAGCG GTGCCAAGA GTTCCATCT GCCAGGTATC
 AGGCAAGGAT ATGGGCTCAC TGAGACTACA TCAGCTATTG TGATTACACC
 CGAGGGGGAT GATAAACCGG GCGCGTGG TAAAGTTGTT CCATTTTTG
 AAGCGAAGGT TGTGGATCTG GATACCGGG AAACGCTGG CGTTAACCAA
 AGAGGCGAAC TGTGTGTGAG AGGTCTATG ATTATGTCCG GTTATGTAAA
 CAATCCGGAA GCGACCAACG CTTGATTGA CAAGGATGGA TGGCTACATT
 CTGGAGACAT AGCTTACTGG GACGAAGACG AACACTTCTT CATCGTTGAC
 CGCCTGAAGT CTCTGATTA GTACAAAGGC TATCAGGTGG CTCCCGCTGA
 ATTGGAATCC ATCTTGCTCC AACACCCCAA CATCTTCGAC GCAGGTGTCG
 CAGGTCTCC CGACGATGAC GCCGGTGAAC TTCCCAGCCGC CGTTGTGTT
 TTGGAGCAGC GAAAGACGAT GACGGAAAAA GAGATCGTGG ATTACGTCGC
 CAGTCAAGTA ACAACCGCGA AAAAGTTGCG CGGAGGAGTT GTGTTGTGG
 ACGAAAGTACG GAAAGGTCTT ACCGAAAAC TCGACGCAAG AAAAATCAGA
 GAGATCCTCA TAAAGGCCA GAAGGGCGGA AAGATGCCG TGTAATTCTA
 GGAATTCCAA CTGAGCGCG GTCGCTACCA TTACCAACTT GTCTGGTGT
 AAAAATAATA GGGGCCGCTG TCATCAGAGT AAGTTAAC TGAGTTCTAC
 TAACTAACGA GTAATATTAA AATTTCAGC ATCTCGCGCC CGTGCCTCTG
 ACTTCTAAGT CCAATTACTC TTCAACATCC CTACATGCTC TTTCTCCCTG
 TGCTCCACC CCCTATTGTT GTTATTATCA AAAAATCTTC TTCTTAATT
 CTTTGTGTTT TAGCTTCTT TAAGTCACCT CTAACAAATGA AATTGTGTC
 ATTCAAAAT AGAATTAACT CGTAATAAAA AGTCGAAAAA AATTGTGTC
 CCTCCCCCCTA TTAATAATA TTCTATCCCA AAATCTACAC AATGTTCTGT
 GTACACCTCT TATGTTTTT TTACTCTGA TAAATTGTT TTGAAACATC
 ATAGAAAAAA CGCACACAA AATACCTTAT CATATGTTAC GTTTCAGTT
 ATGACCGCAA TTTTTATTC TTGCGACGTC TGGGCGCTCTC ATGACGTCAA
 ATCATGCTCA TCGTGAAAAA GTTTGGAGT ATTTTGAA TTTTCATC
 AAGTGAAAGT TTATGAAATT AATTTCCTG CTTTGCTTT TTGGGGTTT
 CCCCTATTGT TTGTCAAGAG TTTGAGGAC GGCCTTTTC TTGCTAAAAT
 CACAAGTATT GATGAGCACG ATGCAAGAAA GATCGGAAGA AGGTTGGGT

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FIG. 3 (CONTINUED 1).

TTGAGGCTCA GTGGAAGGTG AGTAGAAGTT GATAATTGAA AAGTGGAGTA
GTGTCTATGG GTTTTTGCC TTAAATGACA GAATACATTC CCAATATACC
AAACATAACT GTTCTCTACT AGTCGGCGT ACGGGCCCTT TCGTCTCGCG
CGTTCCGGTG ATGACGGTGA AAACCTCTGA CACATGCAGC TCCCAGGAGAC
GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA GCGCGTCAGG
GCGCGTCAGC GGGTGTGGC GGGTGTGGG GCTGGCTTAA CTATGCAGCA
TCAGAGCAGA TTGTAATGAG AGTGCACCAT ATGCGGTGTG AAATACCGCA
CAGATGCGTA AGGAGAAAAT ACCGCATCAG GCAGCCTTAA GGGCCTCGTG
ATACGCTTAT TTTTATAGGT TAATGTCATG ATAATAATGG TTTCTTAGAC
GTCAGGTGGC ACTTTTCGGG GAAATGTGCG CGGAACCCCT ATTTGTTAT
TTTCTAAAT ACATCAAAT ATGTATCCGC TCATGAGACA ATAACCCCTGA
TAAATGCTTC AAAAATATTG AAAAAGGAAG AGTATGAGTA TTCAACATT
CCGTGTCGCC CTTATTCCCT TTTTGCAGG ATTTGCCTT CCTGTTTTG
CTCACCCAGA AACGCTGGT AAAGTAAAAG ATGCTGAAGA TCAGTTGGGT
GCACGAGTGG GTACATCGA ACTGGATCTC AACAGCGGT AGATCCTTGA
GAGTTTCGGC CCCGAAGAAC GTTTCCAAT GATGAGCAGT TTTAAAGTTC
TGCTATGTGG CGCGGTATTA TCCCCTATTG ACGCCGGGCA AGAGCAACTC
GGTCGCCGCA TACACTATTG TCAGAATGAC TTGGTTGAGT ACTCACCAGT
CACAGAAAAG CATCTTACGG ATGGCATGAC AGTAAGAGAA TTATGCAGTG
CTGCCAAC CATGAGTGT AACACTGCGG CCAACTTACT TCTGACAACG
ATCGGAGGAC CGAAGGGAGCT AACCGCTTTT TTGCACAAACA TGGGGGATCA
TGTAACTCGC CTTGATCGTT GGGAACCGGA GCTGAATGAA GCCATACAA
ACGACGAGCG TGACACCACG ATGCCTGTAG CAATGGCAAC AACGTTGCC
AAACTATTAA CTGGCGAACT ACTTACTCTA GCTTCCCGGC AACAAATTAA
AGACTGGATG GAGGCGGATA AAGTTGCAGG ACCACTTCTG CGCTCGGCC
TTCCGGCTGG CTGGTTTATT GCTGATAAAAT CTGGAGGCCGG TGAGCGTGGG
TCTCGCGGT TAATTGCGC ACTGGGGCCA GATGGTAAGC CCTCCCGTAT
CGTAGTTATC TACACGACGG GGAGTCAGGC AACTATGGAT GAACGAAATA
GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG GTAAGTGTCA
GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTA
ATTTAAAAGG ATCTAGGTGA AGATCCTTT TGATAATCTC ATGACCAAA
TCCCTAACG TGAGTTTCGG TTCCACTGAG CGTCAGACCC CGTAGAAAAG
ATCAAAGGAT CTTCTTGAGA TCCTTTTTT CTGCGCGTAA TCTGCTGCTT
GAAACAAAA AAACCACCGC TACCAGCGGT GGTTTGTGG CCGGATCAAG
AGCTACCAAC TCTTTTCCG AAGGTAACTG GCTTCAGCAG AGCGCAGATA
CCAAATACTG TCCCTCTAGT GTAGCGTAG TTAGGCCACC ACTTCAAGAA
CTCTGTAGCA CCGCCTACAT ACCTCGCTCT GCTAATCCTG TTACCAAGTGG
CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CCGGGTTGGA CTCAAGACGA
TAGTTACCGG ATAAGGCGCA GCGGTCCGGC TGAACGGGGG GTTCGTGCAC
ACAGCCCAGC TTGGAGCGAA CGACCTACAC CGAAGTGTGAGA TACCTACAGC
GTGAGCATTG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA GGCAGACAGG
TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC
AGGGGAAAC CCCTGGTATC TTTATAGTCC TGTGGGGTTT CGCCACCTCT
GACTTGAGCG TCGATTTCGG TGATGCTCGT CAGGGGGGGCG GAGCCTATGG
AAAAACGCCA GCAACGCGGC CTTTTACGG TTCTCTGGCCT TTTGCTGGCC
TTTGCTCAC ATGTTCTTTC CTGCGTTATC CCTGATTCT GTGGATAACC
GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCGCAG CGAACCGACC
GAGCGCAGCG AGTCAGTGTAG CGAGGAAAGCG GAAGAGCGCC CAATACCGAA
ACCGCCTCTC CCCGCGCGTT GGCGATTCA TTAATGCAGC TGGCACGACA
GGTTTCCGA CTGGAAAGCG GGCAGTGTAGC GCAACGCAAT TAATGTGAGT
TAGCTCACTC ATTAGGCACC CCAGGCTTAA CACTTTATGC TTCCGGCTCG

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FIG.3(CONTINUED 2).

TATGTTGTGT GGAATTGTGA GCGGATAACA ATTCACACA GGAAACAGCT
ATGACCATGA TTACGCCAAG CTGTAAGTT AAACATGATC TTACTAACTA
ACTATTCTCA TTTAAATTTT CAGAGCTAA AAATGGCTGA AATCACTCAC
AACGATGGAT ACGCTAACAA CTTGGAAATG AAATAAGCTT GCATGCCTGC
AGGCCTGAGA TATTTGCGC GTCAAATATG TTTTGTGTCC CCGTAATATT
TTTTAAATC AAATTCACA TTTTAACCAT AAAAACTCT TTCAAAAGTG
TAATTTCTA CGCAAAATG CCGTCGGAT GAAAAATTAC TTTTGAAAAA
CAAACTCGAA ACTACGGTAC GCAAAAAGT ACATCGGTGT TTGCACATAA
GTGAAAACAA TGTTGTTTT TTGTAATTAA AATCGATTAA TTTTTTTCC
CGGAAAACAA AAACGTTTC AGCGTGGATT TCTATTGTTT CTTGCGTAAA
AAAAAATTAT TTACCAATT TAAACGATAA TTTCCACGAA TTTTCGCCAT
TAATCTCTCG ATTTGTTGA TTCTTGACTC CGAGCAATCT CTCCGGTTT
CGCAACGAT TATATTATT ATTGTTTC CTTTCAGTG CCGATTCTCG
GAAATTCAAC AGTAAATCTT CAAAATGCCA ATGCTTCCCC ACATGGTCAA
TCTAAGTGAG TTTCTTGTT ACAAAATACA CGTGATGTCA GATTGTCTCA
TTTCGGTTTG ATCTACGTAG ATCTACAAAA AATGCGGGAA TTGAGCCGCA
GAGTTCTCAA CTGCTTTCGC ATGGTTAAGA ACGTGCGGAC GTCAAATTGT
TTTGGCAAA AATTCCCGCA TTTTTGTAG ATCAAACCGT AATGGGACAG
TCTGGCACCA CGTGACTATA TATTTTAGC GGTCAACGAC ACAAACCCG
GACCAATGGC TGAGGATCAG CTGAAAGCTT ATAGAGATAG AAATCAGGTG
AGAAAAATCA ATTTCAGCGA TTTCTTCGC AATTATATA AAAACTGATT
TTTCCAGGAA CCCCACCTGC TCACCACATC CAATCGGAGC TCAGAAAAA

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FIG. 4. Nucleotide sequence of pGN156

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FIG. 4 (CONTINUED).

ggcgagcgatacaccgcattccggcgccgattggcctgaactgccagctggcgcaaggtagcagacgcggtaaa
 ctggctcgatttagggccgcaaaaaactatcccaccgccttactgccctgtttgaccgctggatct
 gccattgtcagacatgttagttaaactgtatgtactaactaacaatgtttcatttaatttcagtac
 cccgtacgtttcccgagcggaaaacggctcgctcgccgtcgaaatgtatggccacaccatgt
 gcgccgacttccagttcaacatcagccgtacagtcaacagcaactgtggaaaccagccatcgccatct
 gctgcacgcggaaagaaggcacatggctgaatatcgacggttccatatgggattggcggcactcctg
 gagcccgctcagtatccggaaattccaactgagcggctcgctaccattaccacttgtctgtcaaaa
 ataataagggccgctgtcatcagactaagttaaactgtatgtactaactaacaatgtggaaaccagccatcgccatct
 cagcatctcgccccgtgcctctgacttcaattacttcaacatccatcatgtcttctcc
 tggctccacccttatttttattatcaaaaaacttctttaatttctttagctttagcttctt
 taagtccatcacaatgtggaaattgttagattcaaaaatagaattaattcgtataaaaaagtgcggaaaaaaa
 ttgtgtccctcccccattaataataatttcatccaaaatctacacaatgttctgtttagacttctttag
 ttttttacttctgataaattttttttagaaacatcatagaaaaaccgcacacaaaataccatcatatg
 ttacgtttcagttatgaccgcatttttatttcttcgcacgtctggcctctcatgacgtcaaattcatgt
 catcgtggaaaatgttggatatttttggatatttttcaatcaagtggaaatgtttagaaattatccctg
 cttttgtttttgggggtttcccttattttgtcaagatgttcggggacggcggtttcttgctaaaatca
 caagtattgtatggcggatgcggatgcggaaagatcgaaagaagggtttgggtttaggtttaggtttaggt
 gaagttgataatttggaaatgtggatgttctatgggggttttgccttaatgtacacaaatcccaata
 taccacataactgtttctactagtcggccgtacggcccttctgtctcgccgtttcggtatgacgt
 gaaaaccctctgacacatgcggccggagacggtcacagctgtgttaaggcgatgcccggagcagacaa
 gcccgtcaggcgcgtcugcgggttggcgggttcggggctggcttaactatgcggcatcagacgat
 gtactggagatgcaccatacgccgtgtggaaataccgcacagatgcgttaaggagaaaaataccatcaggc
 ctttaaggccctcgatgcgttattttatgttgcggatcccttgcgttgcgttgcgttgcgttgcgt
 tggactttcggggaaatgtgcggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 gctcatgagacaataaccctgataatgtctcaataatattgaaaaaggaaagatgttgcgttgcgt
 ccgtgtcccttattcccttttgcggatttgccttctgttttgcgttgcgttgcgttgcgttgcgt
 agtggaaatgtgtggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 ctttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 attatccgtatttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 gtactcaccatgtgtggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 catggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 gcacaacatggggatcatgttacttcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 cgacgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 tctagcttcccgcaacaattatgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 cttcccgctggctggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 actggggccatgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 acgaaatagacatgtgtggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 atatataacttttagattttatgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 tctcatgacccaaatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 atcttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 gttttttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 aaataactgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 cgctctgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 acgatagttaccggataaggccggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 aacgacccataccggacttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 ggcggacaggatccggtaaggccggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 ctggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 gggggccggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 tcacatgttcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 cgctcgccgcggccggatcccttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 accggccctcccccgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 cagtggcgcaacgcattatgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
 ggctcgatgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt
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FIG. 5. Nucleotide sequence of pGQ3

CGCGCCATGA GTAAAGGAGA AGAACTTTC ACTGGAGTTG TCCCCATTCT
 TGTGAATT A GATGGTGTG TTAATGGGCA CAAATTTCT GTCAGTGGAG
 AGGGTGAAGG TGATGCAACA TACGGAAAAC TTACCCCTAA ATTATTTGC
 ACTACTGGAA AACTACCTGT TCCATGGGTA AGTTTAAACA TATATATACT
 AACTAACCTT GATTATTAA ATTTCAGCC AACACTTGTC ACTACTTCT
 GTTATGGTGT TCAATGCTTC TCGAGATACC CAGATCATAT GAAACGGCAT
 GACTTTTCA AGAGTGCAT GCCCGAAGGT TATGTACAGG AAAGAACTAT
 ATTTTCAA GATGACGGGA ACTACAAGAC ACGTAAGTT AAACAGTCG
 GTACTAACTA ACCATACATA TTTAAATT T CAGGTGCTGA AGTCAAGTT
 GAAGGTGATA CCCTGTAA TAGAATCGAG TTAAAAGGTA TTGATTTAA
 AGAAGATGGA AACATTCTG GACACAAATT GGAATACAAC TATAACTCAC
 ACAATGTATA CATCATGGCA GACAAACAAA AGAATGGAAT CAAAGTTGTA
 AGTTTAAACT TGGACTTACT AACTAACCGA TTATATTAA ATTTCAGAA
 CTTCAAAATT AGACACAACA TTGAAGATGG AAGCGTTCAA CTAGCAGACC
 ATTATCAACA AAATACTCCA ATTGGCGATG GCCCTGTCTT TTTACCAGAC
 AACCAATTAC TGTCCACACA ATCTGCCCT TCGAAAGATC CCAACGAAA
 GAGAGACCAAC ATGGCTCTT TTGAGTTGT AACAGCTGCT GGGATTACAC
 ATGGCATGGA TGAACATAC AAATAGGGCC GGCGCAGCTC CGCATCGGCC
 GCTGTATCA GATGCCATC TCGCGCCCGT GCCTCTGACT TCTAAGTCCA
 ATTACTCTTC AACATCCCTA CATGCTCTT CTCCTGTGC TCCCACCCCC
 TATTTTGT ATTATCAAA AAACCTCTTC TTAAATTCTT TGTTTTTAG
 CTTCTTTAA GTCACCTCTA ACAATGAAAT TGTGTAGATT CAAAAATAGA
 ATTAATTCGT AATAAAAAGT CGAAAAAAAT TGTGCTCCCT CCCCCCATTA
 ATAATAATTCA TATCCCAAA TCTACACAAT GTTCTGTGTA CACTTCTTAT
 GTTTTTTTA CTTCTGATAA ATTTTTTTTG AAACATCATA GAAAAAACCG
 CACACAAAAT ACCTTATCAT ATGTTACGTT TCAGTTATG ACCGCAATT
 TTATTTCTTC GCACGTCTGG GCCTCTCATG ACGTCAAATC ATGCTCATCG
 TGAAAAGTT TTGGAGTATT TTGGAATT TTCAATCAAG TGAAAGTTA
 TGAAATTAAAT TTTCCTGCTT TTGCTTTTG GGGGTTCCC CTATTGTTG
 TCAAGAGTTT CGAGGACGGC GTTTTCTTG CTAAATCAC AAGTATTGAT
 GAGCACGATG CAAGAAAAGT CGGAAGAAGG TTGGGTTTG AGGCTCAGTG
 GAAGGTGAGT AGAAGTTGAT AATTGAAAG TGGAGTAGTG TCTATGGGT
 TTTGCCCTTA AATGACAGAA TACATTCCA ATATACAAA CATAACTGTT
 TCCTACTAGT CGGCCGTACG GGCCCTTTCG TCTCGCGCT TTCGGTGTG
 ACGGTGAAAA CCTCTGACAC ATGAGCTCC CGGAGACGGT CACAGCTGT
 CTGTAAGCGG ATGCCGGGAG CAGACAAGCC CGTCAGGGCG CGTCAGCGGG
 TGTGGCGGG TGTCGGGCT GGCTTAACTA TGCAGCATCA GAGCAGATTG
 TACTGAGAGT GCACCATATG CGGTGTGAAA TACCGCACAG ATGCGTAAGG
 AGAAAATACC GCATCAGGCG GCCTTAAGGG CCTCGTGATA CGCCTATTTT
 TATAGGTTAA TGTCATGATA ATAATGGTT CTTAGACGTC AGGTGGCACT
 TTTGGGGAA ATGTGGCGG AACCCCTATT TGTGTTTT TCTAAATACA
 TTCAAAATAG TATCCGCTCA TGAGACAATA ACCCTGATAA ATGCTTCAT
 AATATTGAAA AAGGAAGAGT ATGAGTATTCA AACATTCGG TGTGCCCTT
 ATCCCTTTT TTGCGGCATT TTGCTTCTT GTTTTTGCTC ACCCAGAAC
 GCTGGTGAAGA GTAAAAGATG CTGAAGATCA GTTGGGTGCA CGAGTGGGTT
 ACATCGAACT GGATCTAAC AGCGGTAAGA TCCCTTGAGAG TTTTCGCCCC
 GAAGAACGTT TTCCAATGAT GAGCACTTT AAAGTTCTGC TATGTGGCGC
 GGTATTATCC CGTATTGACG CCGGGCAAGA GCAACTCGGT CGCCGCATAC
 ACTATTCTCA GAATGACTTG GTTGAGTACT CACCAGTCAC AGAAAAGCAT

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FIG. 5 (CONTINUED 1).

CTTACGGATG GCATGACAGT AAGAGAATT A TGCA GTGCTG CCATA ACCAT
GAGTGATAAC ACTGC GGCA ACT TACTTCT GACAACGATC GGAGGACCGA
AGGAGCTAAC CGCTTTTTG CACAACATGG GGGATCATGT AACTCGCCTT
GATCGTTGGG AACCGGAGCT GAATGAAGCC ATACCAAACG ACGAGCGTGA
CACACAGATG CCTGTAGCAA TGGCAACAAAC GTTGC GCAA A CTATTA ACTG
GCGA ACTACT TACTCTAGCT TCCCGGCAAC AATTAATAGA CTGGATGGAG
GCGGATAAAAG TTGCAGGACC ACTTCTGC GC TC GGCCCTTC CGGCTGGCTG
GTTTATTGCT GATAAACTG GAGCCGGTGA CGCGTGGGTCT CGCGGTATCA
TTGCAGCACT GGGGCCAGAT GTAAAGCCCT CCCGTATCGT AGTTATCTAC
ACGACGGGGA GTCAGGCAAC TATGGATGAA CGAAATAGAC AGATCGTGA
GATAGGTGCC TCACTGATTA AGCATTGGTA ACTGTCAGAC CAAGTTACT
CATATATACT TTAGATGAT TAAAAACTTC ATTTTTAATT TAAAAGGATC
TAGGTGAAGA TCCTTTTG A TAATCTCATG ACCAAAATCC CTTAACGTGA
GTTTCTGTT CACTGAGCGT CAGACCCCGT AGAAAAGATC AAAGGATCTT
CTTGAGATCC TTTTTCTG CGCGTAATCT GCTGCTTGCA AACAAAAAAA
CCACCGCTAC CAGCGGTGGT TTGTTGCCG GATCAAGAGC TACCAACTCT
TTTTCGGAAG GTA ACTGGCT TCAGCAGAGC GCAGATACCA AATACTGTCC
TTCTAGTGT A GCCGTAGTTA GGCCACCCT TCAAGAACTC TGTAGCACCG
CCTACATACC TCGCTCTGCT AACCTGTTA CCAGTGGCTG CTGCCAGTGG
CGATAAGTCG TGTCTTACCG GGTGGACTC AAGACGATAG TTACCGGATA
AGGCGCAGCG GTCGGGCTGA AC GGGGGGGTT CGTGCACACA GCCCAGCTTG
GAGCGAACGA CCTACACCGA ACTGAGATAC CTACAGCGTG AGCATTGAGA
AAGCGCCACG CTTCCCCAAG GGAGAAAGGC GGACAGGTAT CGCGTAAGCG
GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC
TGGTATCTTT ATAGTCTGT CGGGTTTCGC CACCTCTGAC TTGAGCGTCG
ATTTTTGTGA TGCTCGTCAG GGGGGCGGAG CCTATGGAAA AACGCCAGCA
ACGCGCCCTT TTACGGTT C TGCCCTTTT GCTGGCCTTT TGCTCACATG
TTCTTCCTG CGTTATCCCC TGATTCTGTG GATAACCGTA TTACCGCCTT
TGAGTGAGCT GATACCGCTC GCCGCAGCCG AACGACGGAG CGCAGCGAGT
CAGTGAGCGA GGAAGCGGA GAGCGCCAA TAGC A AACCC GCCTCTCCCC
GCCGGTTGGC CGATTCAATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG
GAAAGCGGGC AGTGAGCGA ACGCAATTAA TGTGAGTTAG CTCACTCATT
AGGCACCCCA GGCTTACAC TTATGCTTC CGGCTCGTAT GTTGTGTGGA
ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG ACCATGATTA
CGCCAAGCTT GCATGCCCTGC AGTGATTCAAGAGGTTGAG ATTATTTTC
AAAAACATTC AATGTTTCC CTGGAGTGA CTATGCAAAT ATGAAAATGT
TTTCCAAAAA TATTTGGATG CCCTGATAAA AAGTAGGTGA AATTTCGAG
GGGAACATCA TATTAATGTT AGAAGAAATG GAAATGTTTG
TCGGTGGTAT GCTCGAATAT TTGAGATATT ATATATTAC TGTAAATCC
GAAATTTTG ACAAACGGAA AAAATTTGTG TCGAAATAC ACATTTCGA
TAACACAAAG GTACTTCCAT AACACTTATA AAAACTGTT GACTATCTTA
ATTGTGTTTC CATGAAGGTA TTGTGAATAT TTTTGACAAA CTGATAGAAT
TTTCAGGAAA AAAAATCCA AGAATAAACAA TTTTCAGAA TTGAACTTT
CTAATGGCTG ATTAATAAAA CAAAGTTATA CAACTATTCA AAGCAGTTGC
TCAATCTGGC ATTTCTTGT GTTTTTTTT GAATATTCA TCAGCAAGAT
GTTGATAATT TTGTGTTAAT TCTAATTGTT TTCTACAATT TTCAAACCG
AAAATGACC TTGACTTTG TTACTTTGT TCTCGGGT TAACTGTTCA
CTGATTCTCA TTGCTGTTGA TGAGGTCTT GATCAAATTT GTATTGTTT
TATACTGCAT ATTGCTTCAA TTCTAAATCA TCTAATATAT TGTCAAACAA
CTTCTTGTGTT TTGTTTCAT TCAAAACTTC TGCAAAACG TTCTCTTAAAC
AAAGGTTCAC ACAACAACTC TCCTCTCCAT CTCTTCTCT CAACAACAAT

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FIG. 5 (CONTINUED 2).

GTGCTGGCCT TGCATGTTG CCAGTGCAGG TTGTTACGC GTTTCAAGA
TTTTGGTCT CCTATCTAAC GTCCCGAAAT GCATTTTC CTTCATTTG
GTTTTTCT GTTCGAGAAA AGTGACCGTT TGTCAAATCT TCTAATTTTC
AGTGAATAAA ATGCTGCAAT CTACTGCTCG CACTGCTTCA AAGCTGGTTC
AACCAGGTTGC GGGGTAAGTC AAAATGAAAT TTTCGTTAA AAATTGGTTT
TTTTGGTAT TATAGATAAA ACTTATACCA AAACAAAACA TATTTAGAAA
AACTTTAATA GAGAATAATT GTTTAATAAT TAATTTTGC AAGCTCCTTT
TAAATTAAGA CATCTAAAC AGTTTCAGC TTGATTGTTT TAATGGTTA
GAAAGCAATA TTTGTATTT GTGTTAAACT GAAAATATCT AGGAAATACT
ACTTTAAAAA TATTTGAAAC TTGAAATTTT AAAATTCCAA ATAATTTAC
TCATTTCTA AAGTGGTGA GTATTTGTAT CCTGTGCTGA CACCGAAATG
TTCTCAATT TGAAAAAAA AGATTTTAT CCGTATCTC AGTCTTACAA
TTTTTTCAC CTTTTTTTC ATTTCAGAGT TCTCGCCGTC CGCTCCAAGC
ACACTCTCCC AGATCTCCC TTCGACTATG CAGATTGGA ACCTGTAATC
AGCCATGAAA TCATGCAGCT TCATCATCAA AAGCATCATG CCACCTACGT
GAACAATCTC AATCAGATCG AGGAGAAACT TCACGAGGCT GTTCGAAAG
GTTTTTTAAT CAGAAGATTT TGAAATGAAT TTTTTTTTG GTATATAGGG
AATCTAAAAG AAGCAATTGC TCTCCAACCA GCGCTGAAAT TCAATGGTGG
TGGACACATC AATCATTCTA TCTTCTGGAC CAACTGGCT AAGGATGGTGG
GAGAACCTTC AAAGGAGCTG ATGGACACTA TTAAGGCTTG G

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FIG. 6. Nucleotide sequence of pGQ4

GTGATTCA GAGGTTGAGA ATTATTTCA AAAACATTCA ATGTTTCGCC
TTGGAGTGC TATGCAAATA TGAAAATGTT TTCCAAAAAT ATTTGGATGC
TGAATTTTA GAAGAAATGG AAATGTTGT CGGTGGTATG CTCGAATATT
TGAGATATTA TATATTTACT GTTAAATCCG AAATTTTGAA CAAACGGAAA
AAATTTGTGT CGAAATACTA CATTTCGAT AACACAAAGG TACTTCCATA
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FIG. 6 (CONTINUED 1).

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FIG. 6 (CONTINUED 2).

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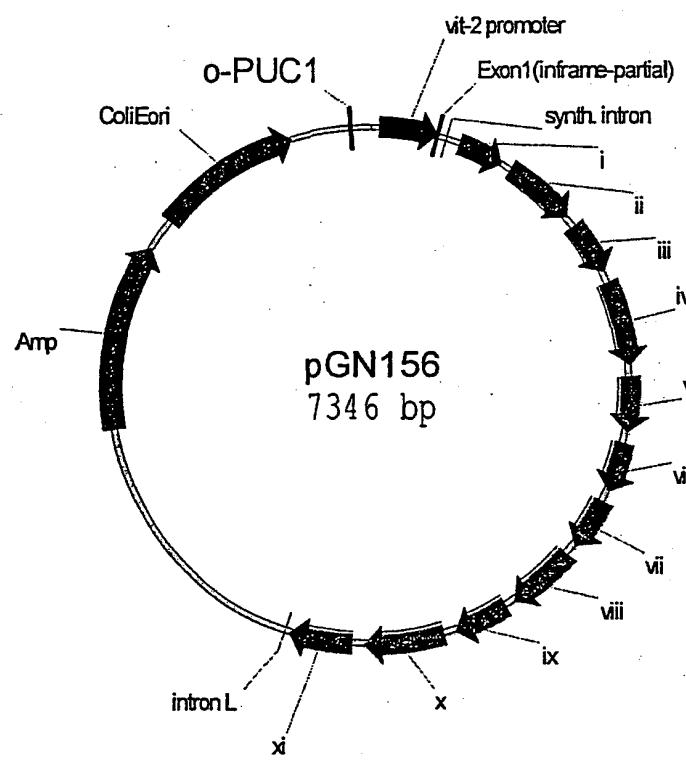
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FIG. 7. Nucleotide sequence of the vit-2 promoter/NLS

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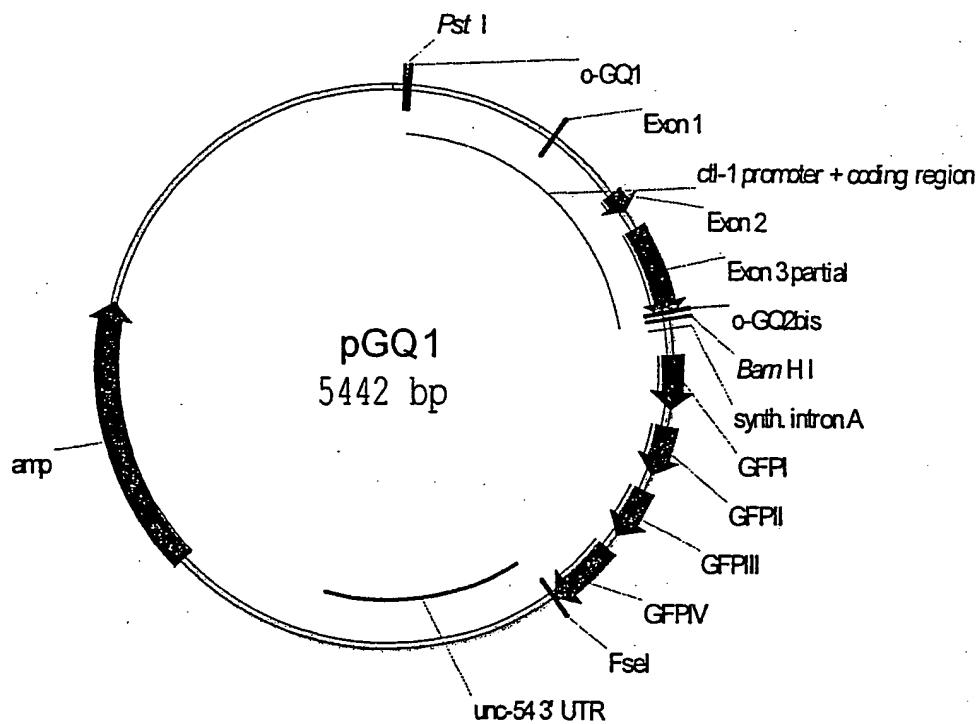
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FIG. 8. Schematic drawing of pGN156



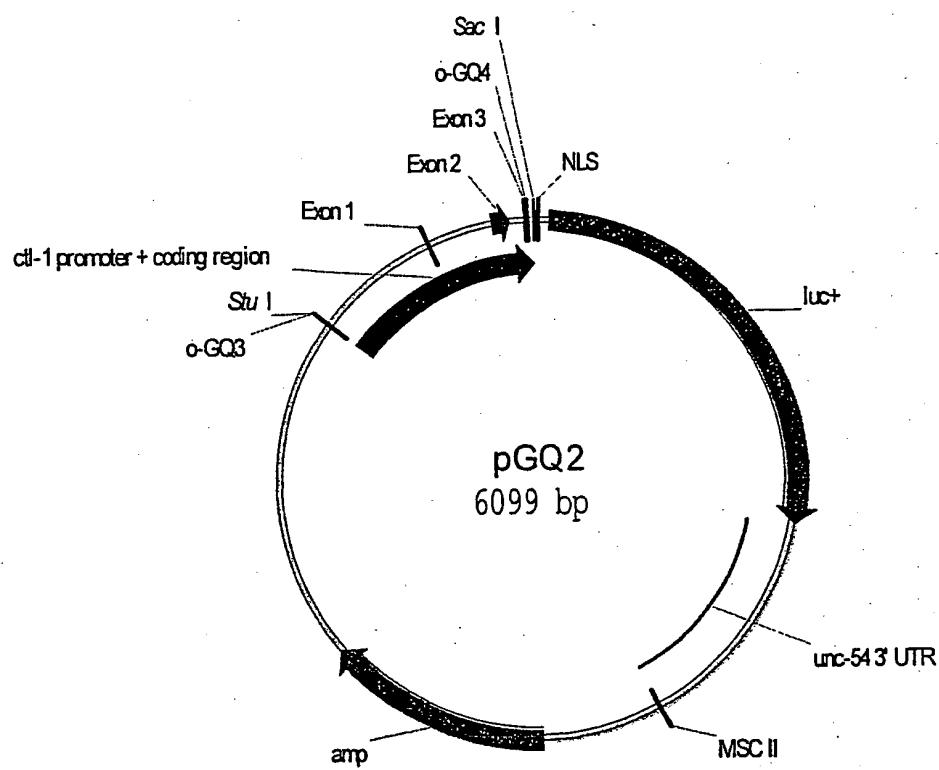
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FIG. 9. Schematic drawing of pGQ1



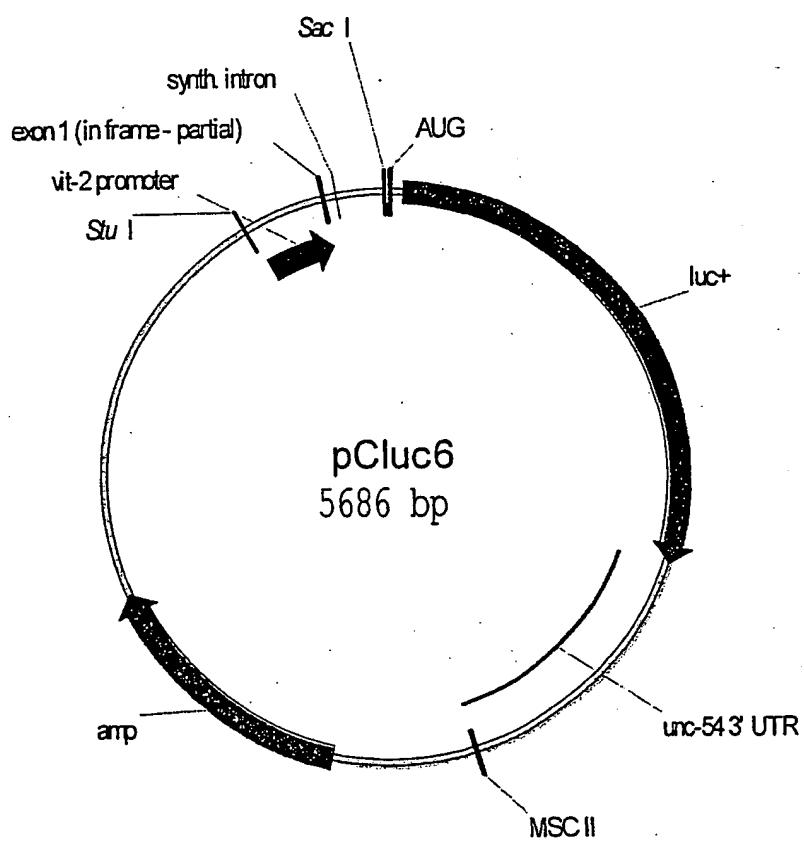
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FIG. 10. Schematic drawing of pGQ2



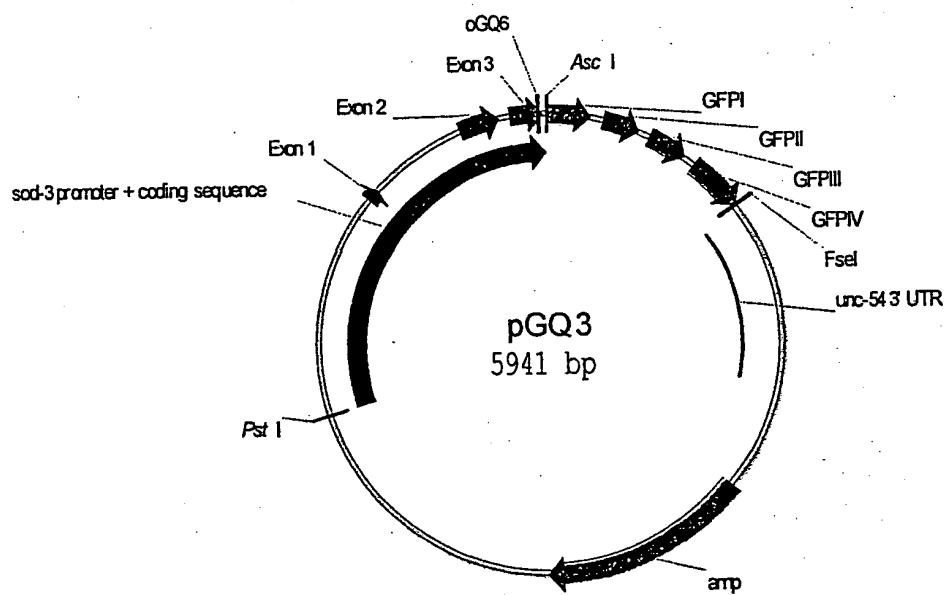
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FIG. 11. Schematic drawing of pCLUC6



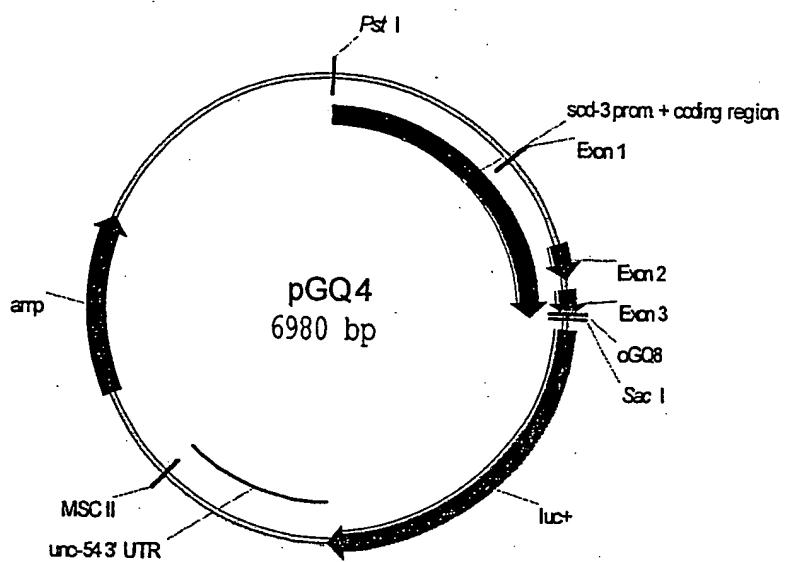
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FIG. 12. Schematic drawing of pGQ3



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FIG. 13. Schematic drawing of pGQ4



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FIG. 14.

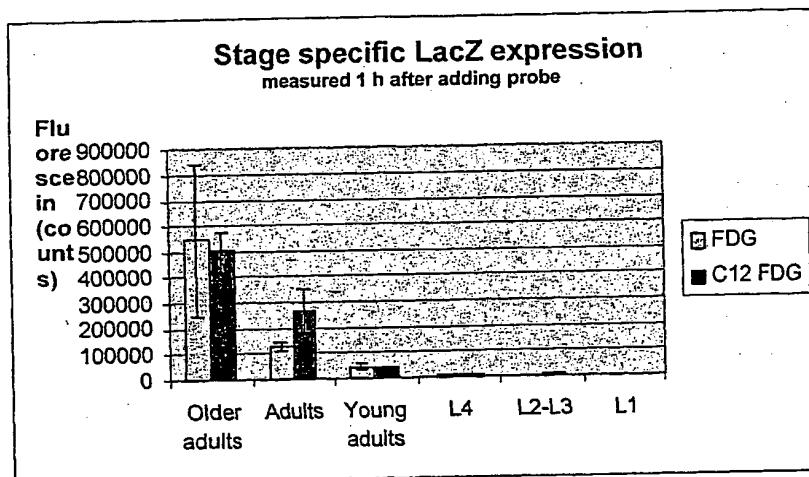
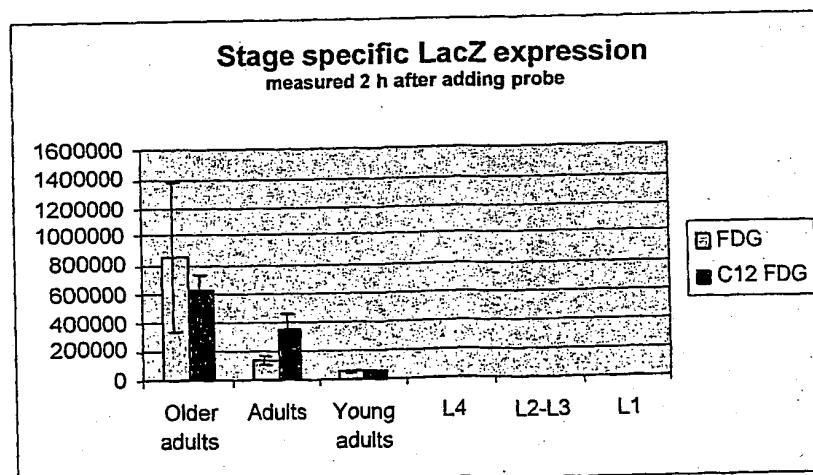


FIG. 15.



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FIG. 16.

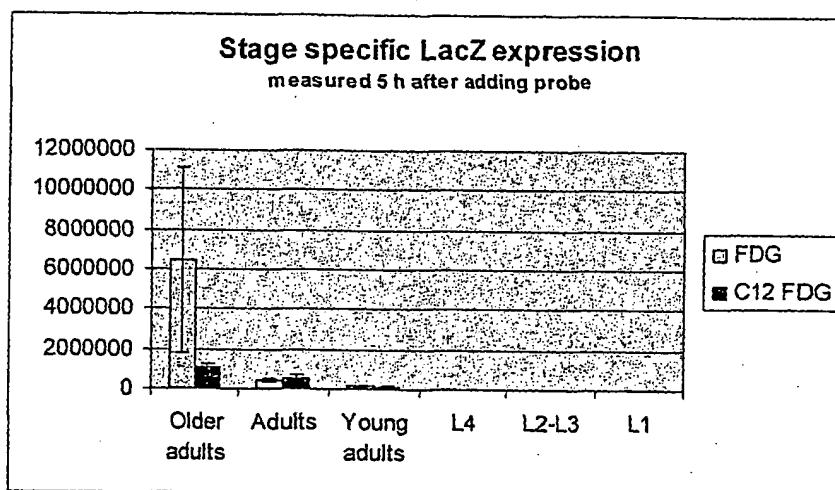
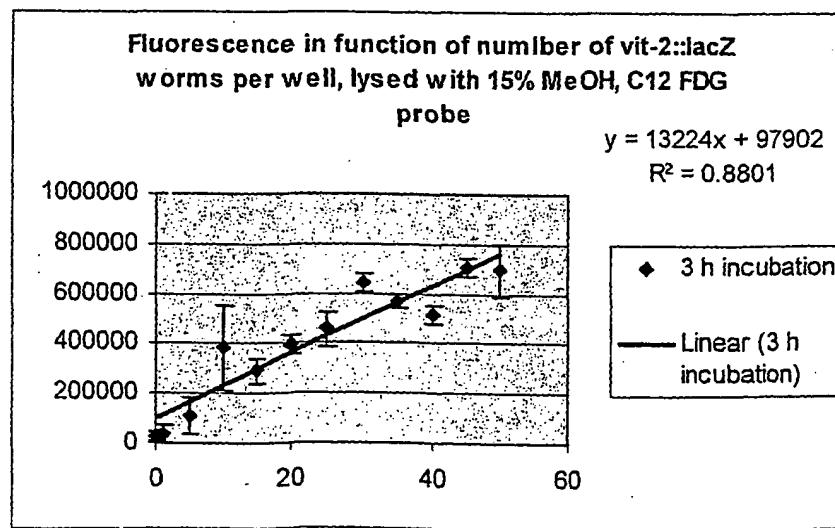
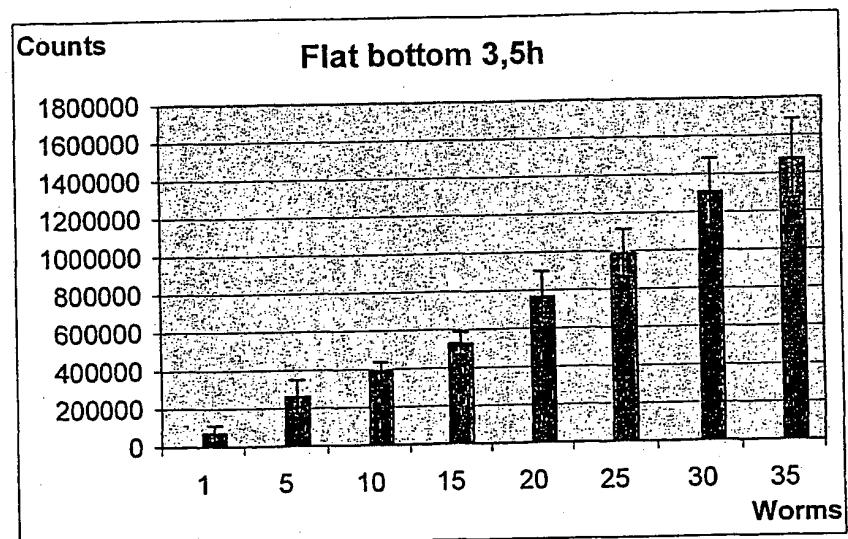
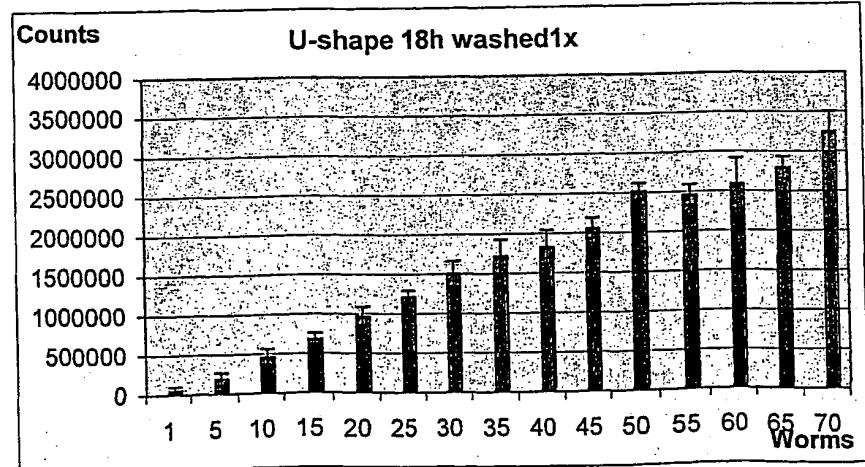


FIG. 17.



*27/27**FIG. 18.**FIG. 19.*

SEQUENCE LISTING

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<130> SCB/57946/001

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Plasmid pGQ2

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Plasmid pGN156

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B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

BELGIAN COORDINATED COLLECTION OF MICROORGANISMS

Address of depositary institution (*including postal code and country*)

Belgian Coordinated Collection of Microorganisms
Laboratorium Voor Molecular Biology - Plasmidencollectie
University of Ghent
K.L. Ledeganckstraat
9000 Ghent, BELGIUM

Date of deposit

01 JUNE 2001

Accession Number

IMBP 5719CB

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 01/01213

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 51351 A (GEN HOSPITAL CORP) 19 November 1998 (1998-11-19) cited in the application the whole document	
A	LIU ZHONGCHI ET AL: "The Caenorhabditis elegans heterochronic gene pathway controls stage-specific transcription of collagen genes." DEVELOPMENT (CAMBRIDGE), vol. 121, no. 8, 1995, pages 2471-2478, XP002191574 ISSN: 0950-1991 abstract	
A	WO 99 01552 A (HESCHELER JUERGEN) 14 January 1999 (1999-01-14) claims 1,11-13	

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Date of the actual completion of the international search

Date of mailing of the international search report

27 February 2002

15/03/2002

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Authorized officer

Niemann, F

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No

PCT/IB 01/01213

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 9851351	A	19-11-1998	US	6225120 B1		01-05-2001
			AU	7494198 A		08-12-1998
			EP	1019092 A1		19-07-2000
			PL	336858 A1		17-07-2000
			WO	9851351 A1		19-11-1998
			HU	0002199 A2		28-09-2000
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			WO	9901552 A1		14-01-1999
			EP	1002080 A1		24-05-2000

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Applicant(s): HOPPE, et al.
Serial No.: 10/766,339
Filing Date: 1/28/2004
Docket No.: DEAV2003/0005 US NP
PRIOR ART

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